Stimuli-Responsive Nanoscale Hydrogels for the Improved Delivery of Chemotherapeutic Agents

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Abstract

Cancer represents one of the largest public health concerns in the world. Current treatment methods for cancer – namely, chemotherapy – possess serious limitations, most notably the nonspecific biodistribution of the cytotoxic drugs, which leads to diminished drug efficacy and undesirable side effects. However, nanomedicines have recently demonstrated great promise in improving the target specificity of chemotherapeutic drugs and thus drug efficacy. Nanomedicines can be designed with ideal properties for drug delivery, and they are able to preferentially accumulate in the diseased site by the enhanced permeability and retention (EPR) effect. However, there are still numerous biological barriers to drug delivery that need to be considered when designing these nanomedicines. In order to overcome these barriers, the nanomedicines or nanoparticles must be rationally designed. By tuning such properties as size, shape, elasticity, hydrophobicity, and surface charge – these nanoparticles can be passively targeted to the diseased site, and even into the cancerous cells. However, it is also beneficial to actively target tumors by functionalizing the particles with target ligands that can preferentially bind to unique or overexpressed receptors or molecules at the tumor site, thereby improving target specificity, as well as facilitating uptake of the nanoparticles by the cell. In the original work of this thesis, the use of pH-sensitive nanogels as drug delivery agents for ovarian cancer was investigated. There were three aims of this investigation: (1) to elucidate the mechanism of internalization in order to determine if endosomal acidification could be leveraged to facilitate cytosolic delivery of the drug payload, (2) to evaluate the effect of stealth coating (PEG) surface density on cellular uptake, (3) and – expecting that the presence of a stealth coating would impede uptake – to design a stimuli responsive peptide linker that would facilitate the shedding of the stealth coating in the presence of a key enzyme that is over expressed in cancer cells – thereby improving uptake. Ultimately, it was determined that the nanogels were internalized via a clathrin-mediated endocytosis process. As a result, these nanogels can, in fact, leverage endosomal acidification to induce nanoparticle swelling, endosomal rupture, and cytosolic delivery. Additionally, particles with no PEG conjugated to the surface exhibited about 90% uptake, but this uptake diminished to about 10% or lower for particles with a PEG content of 10 mol% or higher, showing that the increase in PEG content severely hindered cellular uptake. However, nanoparticles with the enzyme degradable crosslinker that functionalized the PEG to the particle surfaces were successfully synthesized, and it was seen that – in the presence of the enzyme – shedding of the stealth coating was, in fact, observed, and uptake levels returned to those of the particles without PEG coatings. Therefore, it can be concluded that nanogels were rationally designed and successfully synthesized that possessed a multitude of functionalities, each of which contributed to the capacity of the nanogels to delivery drugs to a specific target with a controlled release of the drug payload.

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Dedications

I would like to dedicate this work to my mother, Christine, my father, David, my brother, Travis, and my dearest friends – for their love and support. Through times of both success and failure, they have been there for me, and it has made all the difference.

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Chapter One: Literature Review

Introduction

While the cancer incidence and death rates appear to be declining for both men and women, it remains a major public health problem world-wide. According to American Cancer Society estimates in 2018, there were about 1.7 million new cancer cases with an expected death toll of over 600,000 Americans alone – making cancer the second most common cause of death in the US, exceeded only by heart disease [1]. Globally, about 1 in 6 deaths are due to cancer, with an estimated 9.6 million deaths in 2018. Additionally, the economic impact of cancer is significant, with a total annual cost of nearly $1.16 trillion in 2010 [2].

The current standard of care for cancer patients is generally some combination of surgery, radiation therapy, immunotherapy, and chemotherapy. Chemotherapy involves the application of chemicals or drugs to kill cancer cells in a systemic fashion. While there are numerous types of anticancer drugs, there are common ones that are classified by their mechanism of action, including the following: a) alkylating agents which damage DNA – like Cisplatin and Carboplatin; b) anti-metabolites that replace the normal building blocks of RNA and DNA – like Methotrexate; c) antibiotics that interfere with the enzymes involved in DNA replication – like Doxorubicin; d) topoisomerase inhibitors that inhibit either topoisomerase I or II (like Topotecan or Etoposide, respectively), which are the enzymes involved in unwinding DNA during replication and transcription; e) mitotic inhibitors that inhibit mitosis and cell division – like Paclitaxel; and f) corticosteroids, like Prednisone, which are used for the treatment of cancer and to relieve the side effects from other drugs [3]–[5]. However, for each of the aforementioned drugs, there are numerous limitations, drawbacks, and side effects. One of the primary issues with current chemotherapeutics is the nonspecific cell and tissue biodistribution, resulting in diminished drug

efficacy and undesirable side effects. Additionally, some administered drugs are rapidly metabolized or excreted from the body prior to achieving their intended effect. Therefore, a need arises for a delivery agent that can improve the target specificity and half-life of the drugs [6]–[9].

Recently, nanomedicines have received attention for their potential to be designed in order to overcome the limitations associated with conventional drugs [10]. These nanomedicines or nanocarriers have numerous notable advantages to the current chemotherapeutic agents. They possess unique properties such as their nanoscale size, high surface-to-volume ratio, and favorable physicochemical characteristics; they can affect the pharmacokinetic and pharmacodynamic profiles of drugs to improve the drugs' therapeutic index; and they can improve *in vivo* stability and blood circulation time [6]–[9], [11]. Additionally, nanomedicines can be rationally designed with passive or active targeting functionalities, in addition to stimuli-responsive properties that allow for the controlled release of the drug cargo [11]. Furthermore, Due to tumors' aberrant vasculature, cancerous tissues often exhibit a phenomenon called the enhanced permeability and retention effect, allowing for the preferential accumulation of macromolecules and nanomaterials at tumor sites [6]–[9]. Currently, myriad different compound types of nanomedicines are being studied, including but not limited to albumin-bound nanoparticles, pegylated-liposomes, natural and synthetic polymer-drug conjugates, and various inorganics [11], [12]. While these nanomedicines exhibit great potential for overcoming the various biological barriers imposed against drug delivery, there are still numerous challenges that need to be met. In order to be an effective drug carrier, the nanomedicine must be viable through various stages of circulation, extravasation, accumulation, distribution, endocytosis, endosomal escape, intracellular localization, and ultimately action. Unfortunately, current nanomedicines tend to prove successful in one, maybe two of those drug-life stages. Therefore, careful consideration must be made when

rationally designing future carriers that have the potential of successful delivery, distribution, and accumulation [13]. This thesis describes each stage of a nanomedicine's journey through the body and the current research being performed to improve the drug delivery capabilities of nanocarriers. Furthermore, in this thesis after the literature review, original work from the Peppas lab concerning the design of a stimuli-responsive nanogel with improved cellular delivery and uptake capabilities is presented.

Hallmarks of Cancer and Tumor Biology

In order to understand the goal of rational nanocarrier design, it is first important to understand how tumors – the targets of the carriers – behave and what their characteristics are. Various physicochemical parameters of the nanoparticles affect the nanoparticle-tumor interaction which is crucial for their delivery, uptake, and efficacy. Furthermore, the nanoparticles could be designed to exploit the differences between normal and malignant cells at the cellular and molecular level. Therefore, tumor physiology must be understood in order to better tune the properties of the nanocarriers such that they may overcome the biological barriers.

Tumors develop in a number of ways. Generally, homeostasis occurs within the body to maintain healthy, normal tissue by providing a stable tissue structure and by carefully controlling the proliferation and death of cells. However, through the exposure of the body to carcinogens, mutagens, toxins, radiation, and other infections, tissue homeostasis can be perturbed leading to cellular mutations and tumorigenesis. Furthermore, if the body does not prevent further development of the initial tumor, it may grow uncontrollable, proliferate, and eventually metastasize [14].

Cancer metastasis is a complex multistage process in which cancer cells separate from the initial primary tumor, survive in the circulation of the vascular or lymphatic system, and then seed at distant sites where they may proliferate to form new tumors. In order for metastasis to occur, the cancer cells must possess specific markers, proteins, and factors that can facilitate their invasion of surrounding stroma, their intravasation, and ultimately their extravasation [15].

First, the metastatic cascade is dependent on the loss of adhesion between cells, allowing for the dissociation of the cell from the primary tumor. The cell-to-cell adhesion complex possesses a composition of cell adhesion molecules (CAMs), tight junctions, adherens junctions, gap junctions, desmosomes, and integrins. Therefore, these junctions and binding agents must be disrupted by the cancer cells through various cell pathways of upregulation or downregulation. For instance, HGF/SF – a hepatocyte growth factor – is a cytokine secreted by stromal cells that is known to modulate the expression of tight junction molecules in various human breast cancer cell lines. HGF disrupts the function of the tight junctions in the human breast cancer cells by changing expression of several tight junction molecules at both the mRNA and protein levels [16]. Furthermore, HGF was shown not only to damage the tight junctions, but also to decrease trans-epithelial resistance, allowing for improved paracellular permeability, which is critical to cancer metastasis [17].

In order to invade surrounding stromal tissues and intravasate the circulatory system, the cancer cells must exhibit improved motility. There are numerous compounds that have been discovered that are associated with a process similar to epithelial mesenchymal transition (EMT), wherein cell signaling processes lead to a loss of epithelial character and a gain of mesenchymal character, which has improved migration abilities [15]. One such class of compounds are matrix metalloproteinases (MMPs) which are proteases that cleave cell-adhesion molecules and

extracellular matrix proteins – like E-cadherin – disrupting the cell junctions, loosening cell contacts, and allowing the cancer cells to migrate and invade surrounding tissue and vasculature [15], [18], [19].

In order to travel to new parts of the body, cancer cells must reach and exploit the body's vasculature and lymphatic systems. Cancerous cells exhibit increased expression of proangiogenic factors which leads to neovascularization and the development of tumor microvessels. This tumor vasculature – distinct from healthy tissue – tends to possess structural and physiological abnormalities such as arterio-venous shunts, blind ends, incomplete endothelial linings and basement membranes, and a general lack of smooth muscle or enervation [20]. Consequently, the blood flow in these microenvironments is tortuous, highly irregular, and leakier than that of normal tissues [21]–[23]. Similar to the angiogenesis process, lymphangiogenesis is an important process that is associated with cancer progression and metastasis, as well. Cancer cells tend to overproduce a variety of factors – such as vascular endothelial growth factors (VEGFs) – in order to stimulate both blood and lymphatic vessel growth, which are then exploited by metastasizing cells to circulate within the body [24]. Although cancer cells produce their own vasculature, they also exhibit such rapid, unrestrained growth that it often forces the cells to develop beyond the distance to which oxygen could diffuse. Therefore, the oxygenation of cancer cells becomes highly heterogeneous, leading to tumor hypoxia and even necrotic tissues which can prove harmful to surrounding tissues [25].

As described, in order to metastasize throughout the body and develop secondary tumors, cancer metastases must break from their cell-cell and cell-matrix interactions, invade surrounding tissues, undergo intravasation, survive in circulation, and finally extravasate into a final location and proliferate. Some kind of cell pathway or expression must be altered to facilitate each of these

steps which provides certain targets for therapy. However, not all of the potentially targetable markers for cancer have been identified. Additionally, metastasis in general poses a large problem for cancer treatment. Upon metastasizing, the cancer cells migrate from their primary cancer site to secondary cancer sites, potentially with new properties, which demands the treatment of multiple types of cancers. Additionally, some metastatic events cannot be detected, and certain cancers tend to be asymptomatic, leading to a lack of diagnosis, which allows the metastasized cancer to progress uninhibited [26]. Furthermore, tumors, during or after treatment, can become drug resistant by a number of different mechanisms. In most cases, the resistance is inherent to the cancer, but acquired resistance is also common. Some cancers express an energy-dependent drug efflux pump – known as P-glycoprotein (P-gp), a member of the ATP binding cassette family of transporters – which detects the presence of the anticancer drugs and ejects them [27]. Additionally, there are other forms of drug resistance such as the development of insensitivity to drug-induced apoptosis and the induction of various drug-detoxifying mechanisms [28]. Clearly, there are numerous limitations with current chemotherapeutic approaches and a wealth of metastatic cancer related issues that need to be addressed.

Barriers to Delivery

While designing nanoparticles as drug delivery agents for chemotherapy, it is important to consider the various barriers posed against delivery. The nanoparticles need to do the following things for successful delivery: remain stable in circulation without releasing the drug prematurely, accumulate in the tumor efficiently, and release the drug locally in the tumor tissue or inside the tumor cells. For each of these steps of delivery there could be one or more distinct barriers to overcome.

The drugs must first overcome the first pass effect, wherein the concentration of the drug is reduced due to metabolism in the liver or the gut prior to its reaching the systemic circulation – severely affecting the bioavailability of the drug [29]. This first pass effect can be avoided using alternative routes of drug administration that allow the drugs to be put directly into the systemic circulation, such as intravenous administration. However, the drug can still be removed from the system due to rapid renal clearance in the kidneys if the hydrodynamic diameter of the drug is below a certain threshold.

After being processed and entering the circulatory system, the drugs must avoid clearance from the body by means of opsonization and presentation to the macrophages of the mononuclear phagocytic system (MPS), otherwise referred to as the reticuloendothelial system (RES). During circulation in the blood, opsonins and other blood serum proteins bind to foreign particles by means of various attractive forces, such as van der Walls, electrostatic, ionic, hydrophobic and hydrophilic and others – forming a 'protein corona' [30]. After opsonization, phagocytosis can occur, leading to the removal of the drugs from the system. By binding to the drugs or nanoparticles, the opsonins transition from an inactive to an active conformation that can then bind to specialized cell surface receptors on the phagocytes in order to induce phagocytosis [31]. One popular means of shielding the nanoparticles and drugs from opsonization, and thus the introduction of the particles to the MPS, is through PEGylation. By decorating the particle surface with charge-neutral, hydrophilic PEG through covalent grafting, entrapment, or adsorption, the charge of the surface is altered, and the shielding groups can then block the electrostatic and hydrophobic interactions that facilitate opsonization. Therefore, PEGylation of nanoparticles is a useful means of improving the circulation time of drugs in the bloodstream [31].

After circulation, the nanocarriers that remain must effectively extravasate through the vascular endothelial layer in order to ultimately reach the tumor tissue itself. However, as previously described, a tumor's physiology tends to be abnormal, which makes the nanoparticles' hemodynamics a concern during the designing process. While abnormalities can lead to certain benefits, such as providing gaps in the endothelial lining through which particles can extravasate and accumulate by the EPR effect, the tumor vasculature can also pose certain barriers to drug delivery. For instance, as described, a tumor's abnormal growth rate and behavior can lead to vasculature with chaotic connectivity, heterogenous blood flow, and a high interstitial pressure. These spatial and temporal heterogeneities in blood supply result in a tortuous pathway for diffusion of the nanocarriers into the tumor microenvironment. As such, the spatial distribution of therapeutic agents in tumors is not uniform; uptake is decreased; and successful delivery is difficult [32].

Once within the tumor microenvironment, the nanoparticles must then cross the extracellular-matrix (ECM), which provides structural integrity to the tissue through a cross-linked network of collagen, elastin fibers, proteoglycans, hyaluronic acid, and other structural proteins and components. Depending on the complexity of the matrix, it could potentially increase the resistance to the diffusion of the nanoparticles to the cancerous cells – thereby preventing the therapeutics from having their intended effect [33].

The final barrier to delivery is the cell membrane itself. In order for the therapeutic to successfully bring about cell death, it more often than not needs to diffuse across the cell membrane and be internalized. However, endocytosis of the nanoparticle is mediated by a variety of different cell pathways and can be affected by numerous properties of the nanoparticles, such as surface charge, size, hydrophobicity or hydrophilicity, shape, flexibility, coating, and ligand density – as

each of these properties will affect the nanoparticles' interactions with the cell membrane and its receptors. Additionally, in crossing the membrane, the nanoparticles become encapsulated in an endosomal vesicle, and the intracellular sites or organelle to which the cargo is trafficked is pre-determined, signal-dependent, and critical to effective therapy [34]. The nanoparticles, thus, must escape from the endosome or the lysosome to which the endosome fused in order to be dispelled into the cytoplasm and cause their cytotoxic effects. There are numerous classes of nanoparticles that achieve this effect in various ways. For instance, some materials like fusogenic lipids undergo phase transitions due to the acidic pH environment of the endosomes or lysosomes, which allows them to then fuse with the membrane and enter the cytoplasm [35]. Even after escaping into the cytoplasm, though, only a partial fraction of the original drug dosage can affect cell death because some of the drugs are recycled back out of the cells. Some tumors implore drug efflux transporters to prevent compounds that are dangerous to the cells from exerting their intended effect [36]. Clearly, there are numerous barriers to overcome in order for nanoparticle drug carriers to prove effective in intracellular delivery.

Mechanisms of Internalization

As previously mentioned, cells possess multiple pathways through which materials like nanoparticles can be internalized, namely pinocytosis – which includes macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis – and phagocytosis, which involves the engulfing of large particles. It is crucial to understand the mechanism by which nanoparticles used for drug delivery are internalized in order to better tailor their properties for improved uptake. In this section, each mechanism and the factors that determine its use will be described.

Both phagocytosis and macropinocytosis are actin-dependent mechanisms that result in the internalization of particles or materials in the micrometer size range [37]. During phagocytosis, a phagocyte, such as a macrophage, will interact with the particle via a receptor and trigger uptake by the formation of a phagosome [38]. Macropinocytosis similarly involves the engulfment of particles in addition to a large amount of extracellular fluid through to formation of membrane protrusions that ultimately recombine with the cell membrane forming a macropinosome [37].

Clathrin-mediated endocytosis – also referred to as receptor-mediated endocytosis – is a cellular uptake mechanism during which the binding of a ligand on the particle to the membrane surface receptors triggers the formation of clathrin-coated endocytotic vesicles approximately 100 nm in diameter [39]. The clathrin assembly induces the vesicle formation initiation as well as the vesicle necking and pinching after successfully wrapping of the membrane around the material [37]. Release of the vesicle from the plasma membrane occurs due to the activity of a GTPase called Dynamin, and these released vesicles either become recycling endosomes or early endosomes that ultimately differentiate into mature endosomes or bind with lysosomes – causing the contents to endure an acidification process along the way [40]. Caveolin-mediated endocytosis involves the assembly of caveolin coats on the cytosolic side of the cellular membrane, resulting in the formation of flask-shaped pits approximately 50-100 nm in diameter [37], [41]. The most notable use of caveolin-mediated endocytosis by cells is for lipid homeostasis – particularly the internalization of cholesterol [41]. One important distinction between clathrin-mediated and caveolin-mediated endocytosis is that the caveosomes possess a neutral pH and avoid the acidification that the endosomes created by clathrin-mediated endocytosis endure [40]. While distinct in their manner of formation, it is known that both clathrin-mediated and caveolin-mediated endocytosis require complex signaling cascades that have yet to be fully understood

[42]. While these two endocytotic pathways are the primary means by which nanoparticles tend to be internalized, clathrin- and caveolin-independent endocytosis have also been observed, but the mechanisms by which materials are uptaken in these manners remain poorly understood and generally described only using negative terms relative to the previously described mechanisms [41].

Intracellular Trafficking

Once a nanoparticle or material has been internalized by the cell by some mechanism, it must be trafficked to its final destination. Additionally, if the lysosome or the endosome is not the final target of the therapeutic, then the nanocarrier must escape from the endosome in order to impart the intended effect on the cell. As previously described, the endosomes that encapsulate the nanoparticles can have various fates. It is possible that some will be recycled to the plasma membrane, thereby releasing the drugs back into the extracellular environment; some will differentiate into mature endosomes that fuse with lysosomes containing hydrolytic enzymes that degrade the nanoparticles; and some might carry the drugs to the targeted cellular component or release the drugs into the cytoplasm due to some functionality inherent to the nanoparticle [43]. Due to the variety of mechanisms that can be induced to cause the release of the nanoparticles from the endosomes, it is useful to look at a few examples of intracellular trafficking, and how designing the nanoparticles appropriately is crucial to their successful use as therapeutic agents.

Some polyamine-based nanocarrier systems have shown promise for controlled intracellular delivery by exploiting the hypothesized “proton sponge” effect. Once internalized by an endosome, the carriers become protonated within the acidic environment. As the protons accumulate together with their counter ions, an osmotic pressure imbalance is created between the endosome and the cytosol. This imbalance stimulates the entrance of water from the cytosol into

the endosome, forcing the endosome to swell and ultimately rupture – leading to the distribution of the endocytosed nanoparticles into the cytoplasm [44].

Some therapeutics require delivery to a specific organelle or region within the cell, rather than simply being distributed to the cytosol. For instance, some therapeutics target and must be delivered to the mitochondria of the cell. Due to the selective and impermeable nature of the mitochondrial membrane, a specially designed vector must be used to successful ferry small molecule drugs to the organelle [45]. Triphenyl phosphonium (TPP) cation – because of its delocalized positive charge and lipophilicity, has proven successful as a carrier for covalently conjugated small molecule drugs for this purpose [46].

Similarly, the design of the nanocarrier must be considered when attempting to target and deliver therapeutics to the nucleus. In general, transport across the nuclear envelope occurs in two ways: passively, most notably for small ions and macromolecules, and actively, facilitated by the binding of oligopeptide sequences to nuclear receptors, known as nuclear localizations signals (NLSs) [40]. There has been success in delivering nanoparticles directly to nucleus using conjugated NLS based carriers. In fact, NLS-functionalized DOX-loaded PLGA nanoparticles were shown to be more successful in delivering drugs to the nucleus of MCF7 cells, relative to their non-NLS conjugated particle counterparts [47].

Though, there are many other strategies for targeting the nucleus of a cell as well – one of which is caveolae-mediated nucleus targeting. As previously described, caveolae-mediated endocytosis is distinct from clathrin-mediated endocytosis and other endocytic pathways in that its caveosomes do not possess an acidic environment, but rather a neutral one – thereby bypassing the acidic, as well as enzymatic, degradation of the internalized components. Additionally, the cargo of the caveosomes are often directed toward the Golgi Apparatus (GA) or the Endoplasmic

Reticulum (ER), which are organelles known to process and redirect cargoes, like proteins and macromolecules, from those organelles to other parts of the cell, including the nucleus. Attempts have been made to target caveolin-mediated endocytic pathways in order to bring the particles to the GA or the ER in the hopes that they will facilitate transport to the nucleus [48]–[50]. In a similar vein, attempts have been made to implore saccharide functionalities into polymer nanocarriers to mimic the natural process of transporting glycosylated proteins from the ER to the nucleus in order to improve delivery [51].

Passive Targeting

Designing nanomedicines for targeted intracellular delivery is crucial in order for the carried drugs to impart their intended effect on the diseased cell. However, it is equally important that the nanoparticles are designed to reach the diseased site in the first place. Regarding drug delivery via nanocarriers, there are two types of targeting that must be considered while designing the nanoparticles: passive and active. Passive targeting takes advantage of the pathophysiological features of the tumor microenvironment in order to facilitate successful accumulation at the diseased site, while active targeting generally relies on the binding of a target ligand to a target receptor in order to further enhance target specificity and facilitate internalization of the nanoparticles. These two strategies of targeting have been illustrated below in **Figure 1** [52]. As previously described, due to the leaky vasculature and the impaired lymphatic drainage inherent to tumors, nanoparticles and macromolecules preferentially accumulate in tumors due to the EPR effect. Therefore, what is of the most importance to consider is those characteristics of the nanoparticles that allow for the exploitation of this effect and further enhance successful accumulation and uptake – namely nanoparticle size, shape, elasticity, and surface chemistry.

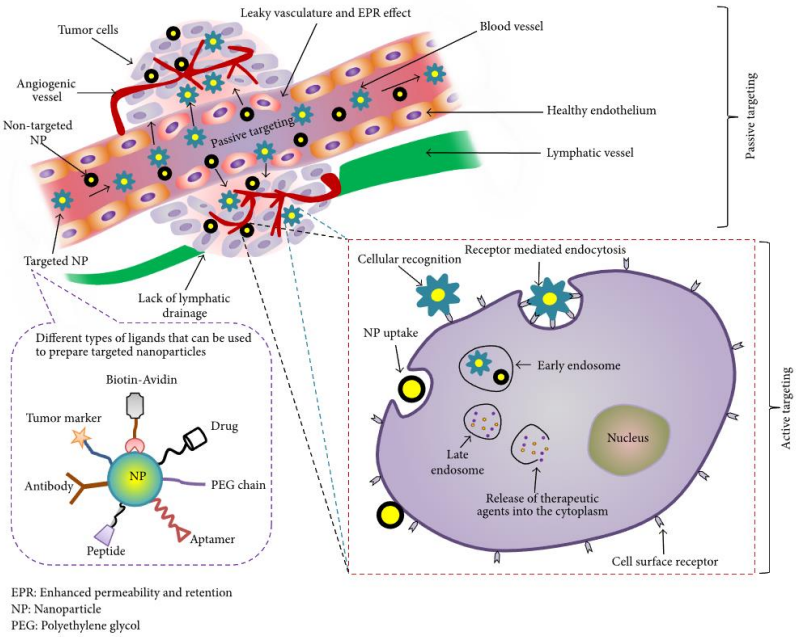

Figure 1. *Schematic representation of both passive and active targeting approaches to enhancing the drug delivery capabilities of a nanocarrier.*

The size of the nanoparticles affect how they interact with the cell membrane and thus the efficiency with which they are uptaken, as well as the mechanism by which they are uptaken. As previously mentioned, particles approaching and in the micron size range are internalized by micropinocytosis and phagocytosis, and particles in the 50-200 nm range tend to be internalized by clathrin- or caveolin-mediated endocytosis. In fact, several studies have determined that, on average, the optimized particle size for cellular uptake is approximately 50 nm. Particles of that size tend to more efficiently bind with cell membrane receptors and thus facilitate receptor-mediated endocytosis [53]–[55]. Aside from affecting uptake – its rate and mechanism – size also affects the particle's circulation. As particles surpass a hydrodynamic diameter of approximately 200 nm, their clearance rate by the reticuloendothelial system increases, thereby reducing the carriers' bioavailability at the target [43], [56].

Though, size alone does not determine which mechanism is employed for internalization, nor does it determine efficiency. In fact, in studying the relationship between nanoparticle size and cellular uptake, results from one study are often found to be inconsistent with those of another study [43], [57]–[59]. These contradictions are most likely due to the sheer number of factors that influence cellular internalization, and the inherent difficulty in unconfounding the effects of each factor on uptake. Additionally, there could be unforeseen or undetectable issues that affect internalization, such as agglomeration or aggregation. Furthermore, cellular uptake varies between cell type. For instance, while embryonic fibroblasts preferentially internalize gold nanoparticles of 25 nm, rather than larger nanoparticles, epithelial cells preferentially internalized 50 nm gold nanoparticles, rather than those larger or smaller [53], [60]. Therefore, when designing a nanoparticle to serve as a drug delivery agent, multiple factors must be considered together in order to provide a synergistic effect in improving delivery and efficacy.

As previously mentioned, other factors affect internalization – like shape. However, similar to the reports on the effect of size, there is little consensus on the role of shape on internalization. In one study performed by Chithrani et al. on the effect of shape on the uptake of gold nanoparticles by HeLa cells, the spherical particles were uptaken five times as much as their rod shaped counterparts [60]. On the other hand, in one study by Gratton et al. regarding uptake of monodisperse hydrogel nanoparticles by HeLa cells, it was found that rod-shaped nanoparticles were internalized at much higher rates than the sphere, cylinder, and cube shaped counterparts [57]. Additionally, in a study by Xu et al. regarding layered double hydroxide nanoparticles with fluorescein isothiocyanate in various morphologies, it was shown that, while the mechanism was the same for the various morphologies, the intracellular trafficking was different [61]. Overall, the

shape of the nanocarrier must be tailored for the specific class of material, for the target cell type, and for the internal destination within the chosen cell.

Yet another factor inherent to the nanoparticles that can affect uptake is the material's elasticity. The most common parameter used to measure a material's elasticity – or its stiffness or flexibility – is Young's modulus, wherein a material with a high Young's modulus is stiff. According to a study by Anselmo et al., it was discovered that softer nanoparticles allowed for improved circulation times and thus enhanced targeting compared to harder nanoparticles *in vivo*; however, the softer nanoparticles exhibited reduced cellular uptake [62]. In an energetics study of cell-nanomaterial interactions using a theoretical model, Yi et al. showed that endocytosis is highly dependent on the stiffness of the nanoparticle. Yi et al. explained that softer nanoparticles were more difficult to be internalized by a cell due to the larger wetting angle inherent to soft materials and the higher extent of spreading by the nanoparticle on the cell membrane. This increased spreading creates a large energy barrier to cell membrane wrapping that impedes, and potentially even stops, cellular uptake [37], [63]. Therefore, the elasticity of a nanocarrier must be tuned such that it is optimized for both ideal circulation and uptake behavior.

One of the most important characteristics of the nanoparticle is its surface chemistry – its charge, hydrophobicity/hydrophilicity, and whether it possesses a stealth coating. The surface chemistry affects the nanoparticle-cell membrane interaction and the uptake mechanism, as well as the nanoparticle-opsonin interaction during circulation. According to both *in vitro* and molecular dynamics simulations, both negatively and positively charged particles have better adhesion to cell membranes and thus internalization than neutrally charged particles [64], [65]. Though, the type of charge affects the membrane of the cells differently. Positively charged particles often lead to enhanced fluidity of the cell membrane. On the other hand, negatively

charged particles can cause gelation of the cell membrane [66]. Additionally, as charge density increases, the energetics of membrane wrapping decrease, improving the ease of internalization. However, an increase in charge density can lead to severe disruption of the cell membrane upon penetration of the cell by the particles [67]. Therefore, the charge density on a particle needs to be optimized for uptake as well as cytotoxicity.

The hydrophobicity or hydrophilicity of the nanoparticles also affects the interactions of the particles with the cell membrane in a similar manner to that of the nanoparticle surface charge. Particles that are hydrophobic tend to become embedded in the hydrophobic core of the lipid bilayer. On the other hand, particles that are hydrophilic tend to adsorb to the cell membrane surface, inducing membrane wrapping. As such, the surface hydrophobicity or hydrophilicity of the nanoparticles can induce different response mechanisms by the cells with which they interact [68].

As previously described, it is known that there are advantages to grafting hydrophilic stealth coating agents – like poly(ethylene glycol) (PEG) – to the surface of nanoparticles for use in biological applications. They shield the cationic particle surface, thereby improving the retention of colloidal stability in physiological conditions; they prevent opsonization by serum proteins, reducing uptake by macrophages of the mononuclear phagocyte system (MPS); and they further enable extravasation by enhancing the circulation time and thus the time to exploit the EPR effect. However, the inclusion of a surface graft can also interfere with the ability of the nanoparticles to interact with the cell membranes. As previously described, while hydrophobic surfaces readily interact with the lipophilic membrane of cells, facilitating cellular uptake, hydrophilic surfaces – like those of PEG-conjugated nanoparticles – do not because the required membrane wrapping for endocytosis of hydrophilic materials creates an energy barrier that must

be overcome, thus impeding uptake by cells [69]. Therefore, while designing a nanoparticle with ideal properties for passive targeting, the surface chemistry and functionalities must be carefully considered – in conjunction with the numerous other factors that affect delivery and uptake.

Active Targeting

Passive targeting is crucial for facilitating efficient accumulation of nanoparticles in the tumor interstitium and ensuring that the nanoparticle itself does not impede uptake. However, more steps need to be taken in order to further promote uptake by the cancer cells. If uptake is not readily and sufficiently achieved, it is possible for the drugs to diffuse out of the tumor site, leading to an undesirable exposure of the cytotoxic drug to healthy tissues. The promotion of uptake can be facilitated by actively targeting the nanoparticles to receptors, proteins, or other components that are either unique to cancer cells or overexpressed in them. This active targeting is most often achieved by functionalizing the surface of the nanoparticles with a key targeting ligand. Due to the uniquely abnormal behavior of cancer, there are numerous different targets that can be considered. These targets can be localized in the tumor microenvironment and extracellular matrix, on the surface of the cancerous cells, or within the cell. Each environment possesses multiple targets of interest.

In the tumor microenvironment and extracellular matrix, some targets include vascular endothelial growth factors (VEGF), vascular cell-adhesion molecules (VCAMs), matrix metalloproteinases (MMPs), and integrins – each of which were described or mentioned previously in this work. VEGF is highly overexpressed in tumor cells, relative to healthy cells, and it promotes tumor angiogenesis and cancer cell proliferation. Additionally, it possesses immunosuppressive capabilities – inhibiting the function of T cells, increasing the recruitment of regulatory T cells and myeloid-derived suppressor cells, and hindering the differentiation and activation of dendritic

cells. As such, VEGF and its receptor, VEGFR, have become key targets for restoring antitumor immunity and for impeding the development of tumoral vasculature [68]. Currently, there are multiple phase I/II clinical trials using monoclonal antibodies to target VEGF and VEGFR pathways [70]. One of these antibodies is Bevacizumab, which binds to VEGF-A and prevents its binding with VEGFR1 and VEGFR2 [71]. Additionally, there are numerous tyrosine kinase inhibitors which prevent the phosphorylation of VEGFR – thereby blocking the signaling cascades that would lead to the stimulation of angiogenesis [72]. Incorporation of these antibodies and inhibitors with delivery agents could significantly improve their efficiency, as well as allow for the dual delivery of the inhibitor and other chemotherapy drugs to the tumor site – providing a synergistic effect for the delivered therapies.

VCAM-1 is a cell adhesion molecule that was previously known to regulate inflammation-associated vascular adhesion and the transport of leukocytes like macrophages and T cells. However, similar to VEGF, in cancer, increased VCAM-1 expression has been linked to tumoral angiogenesis and metastasis [73]. Again, similar to the targeting of VEGF and VEGFR, there have been a litany of different peptides and antibodies used in various studies that have been functionalized to nanoparticles for the targeting of VCAM-1 for both diagnostic and therapeutic purposes. In general, in these approaches, the VCAM-1 is exploited as a marker of the diseased site, and the binding of the antibodies or peptides to VCAM-1 facilitate the preferential accumulation of the conjugated nanoparticles carrying drugs in the diseased site [74]–[78].

MMPs are a family of zinc-dependent endopeptidases that are utilized for the remodeling of the extracellular matrix. As such, they are often exploited by and overproduced in cancer cells in order to cleave necessary cell-adhesion molecules – thereby allowing cell invasion, tumor growth, and ultimately metastasis [79]. Several attempts have been made to exploit the presence

of these proteinases in tumors. In one study designed, an MMP 2-responsive multifunctional liposomal nanocarrier was designed for enhanced tumor targeting. In this case, an MMP-2 cleavable peptide served as a linker between the liposomal surface and the surface grafted stealth agent, PEG, which is known to impede uptake. As such, the cleaving of the peptide linker facilitated the cellular uptake of the liposomal nanocarrier carrying cytotoxic drugs that would then cause the death of the cancer cell [80]. A similar approach was used in another study, in which the MMP substrate peptide was conjugated to the surface of mesoporous silica nanoparticles containing cytotoxic drugs. Again, the cleavage of the MMP substrate facilitated cellular uptake and ultimately led to cell death – showing the improved target specificity for the MMP-expressing tumor site [79], [81].

Integrins are transmembrane receptors that serve as cell-surface adhesion molecules, and they are known for their bidirectional signaling capabilities – integrating processes that occur in the intracellular compartment with those of the extracellular environment [82]. As such, integrins – through mechanosensing and force-transduction signaling – play a special role in regulating important functions like proliferation, gene expression, cell survival, and cell mobility [83]. However, cancer cells often exploit these integrin-mediated processes in order to create an environment that promotes cancer progression. In fact, the deregulation of integrin-mediated cell adhesion to the extracellular matrix is a crucial step to tumorigenesis – facilitating proliferation, invasion, and ultimately metastasis. Therefore, disrupting this exploitation is a key route towards treating cancer and preventing its growth [84]. Efforts have been made to target the activity of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins – key components associated with regulating angiogenesis and tumor growth – directly by using the inhibitor, Cilengitide, and they have shown great promise [85]–[88]. Though, there are also efforts being made to create integrin-targeted medicines that deliver cargoes

such as chemotherapeutics, proapoptotic peptides, and radionucleotides to the tumor cells and the surrounding vasculature [86]. For instance, Hood et al. demonstrated the successful delivery of a mutant *RAF1* gene to a tumor site using an integrin αvβ3-targeted nanoparticle that ultimately resulted in the apoptosis of the cancer cells and the regression of the tumor – demonstrating the effectiveness of targeted nanoparticles for drug delivery purposes [89].

As previously mentioned, there are also many active targeting strategies that involve cell surface receptors that are either uniquely expressed or overexpressed in cancer and the use of one of a variety of targeting ligands, such as antibodies, proteins, aptamers, peptides, carbohydrates, and other small molecules. One of the many receptors that is known to be overexpressed in a variety of epithelial cancers is the epidermal growth factor receptor (EGFR) [90]. One of the most commonly used targeting ligands for EGFR is Cetuximab, which is a chimeric human-murine monoclonal antibody that competitively binds to EGFR [91]. Cetuximab has been used to facilitate the targeted delivery of gold nanoparticles to EGFR-positive cancer sites for both therapeutic and diagnostic purposes in a multitude of different studies [92]–[97].

The transferrin receptor is another cell receptor that is often targeted for various cancer therapies. While the transferrin receptor is expressed on most normal human tissues, it is expressed on malignant cells at levels several fold higher than those of normal cells due to the necessity of iron (delivered by the protein, transferrin) for cancer cell proliferation [98]. One method of targeting this receptor is through the use of monoclonal antibodies that antagonize the normal function of the receptor and induce cytotoxic effects [98]–[102]. Another method of targeting this receptor is through the conjugation of transferrin – the targeting moiety and primary receptor ligand – directly to the active compound that will facilitate cell death or to a nanocarrier that contains the active compound [98]. The targeting of this receptor can prove particularly useful for

cancer treatments due to its aforementioned high levels of expression and because of the wealth of possibilities of things that can be and have been conjugated to the protein, transferrin, including a litany of chemotherapeutics, toxic proteins, peptides, nucleic acids, antibodies, and carriers [98].

As previously mentioned, aptamers are often used in cell surface targeting strategies for drug delivery systems. Aptamers are single-stranded DNA or RNA oligonucleotides that form secondary and tertiary structures that can bind to proteins and other cellular targets with high specificity, much like antibodies [103]. Aptamers are produced through an iterative *in vitro* selective process called "systemic evolution of ligands by exponential enrichment" (SELEX) [104]. During this process, a large random sequence library of ssDNA or ssRNA molecules are exposed to the target molecule, with the expectation that a small subset of the aptamers will possess the capability to fold in such a way that they may bind to the target molecule with high specificity. The non-binding aptamers are then disposed of, and the aptamers that specifically bind to the target molecule with high affinity are enriched by a sequential selection round of PCR or RT-PCR amplification. This process is iterated – gradually creating aptamer libraries of increasing binding affinity – until the best candidate aptamer(s) for the specific target is selected [103], [104]. Due to the high binding specificity of the targeting moieties created through this SELEX process, aptamers can serve and have served as powerful tools for actively targeting overexpressed receptors on cancer cells and facilitating the transport of their conjugated cargoes to diseased sites [105]–[110].

Certain carbohydrates have also recently received attention for serving as targeting moieties for cancer cells – one of which is mannose. The receptor for mannose, MR is often over expressed in many tumors because cancer cells exhibit a heightened affinity for carbohydrates, relative to that of normal cells, in order to supply the high demand of nutrients necessary for the

rapid proliferation of the cancer – making it a reliable target for drug delivery. Additionally, although cancer cells exhibit high tendencies to mutate and develop resistance mechanisms, the MR has been shown to be a relatively stable marker, making drug delivery to MR over-expressing cells more predictable. As such, multiple mannose-functionalized tumor-targeted drug delivery systems have been developed and have shown great promise for improving drug efficacy [111]–[115].

Yet another molecule considered for targeted delivery strategies is folic acid, which is a small molecule vitamin used by eukaryotic cells to synthesize purines and pyrimidines [116]. Its uptake is primarily facilitated by a low-affinity-reduced folate carrier. It is also uptaken via the high affinity glycosylphosphatidyl-inositol-linked folate receptor (FR), although not to the same level as that of the reduced carrier [117], [118]. However, while the carrier only transports reduced forms of folic acid, FR is capable of transporting non-physiologic forms of the vitamin into cell, as well as folate-conjugated nanoparticles [117]. While only minimally expressed in healthy tissues, the FR is over expressed in many tumors due to the need of the cancer cells to supply the nutrients necessary for DNA replication and thus cancer proliferation and tumor growth [119], [120]. As such, a significant amount of research has been devoted to the creation of folate-conjugated nanocarriers to facilitate improved target specificity for cancer sites [121]–[128].

In the previous section of this work on intracellular trafficking, a few strategies were discussed concerning the controlled delivery of cargoes to specific organelles. These strategies can be considered methods of active targeting. Though, there are also other ways to actively target intracellular components of cancer cells, as there are a multitude of compounds that are overexpressed in cancer cells. For instance, cyclooxygenase-2 (COX-2) is one of a family of enzymes which catalyze the rate-limiting step in the biosynthesis of prostaglandins, which are

proinflammatory lipids. The cyclooxygenase family of enzymes, including COX-2, are located at the luminal side of the endoplasmic reticulum and nuclear membrane within the cell [129]. COX-2, which is the inducible isoform of the enzyme, is known to be upregulated during both inflammation and cancer. The induction or overexpression of COX-2 is associated with an increased production of PGE2, which is known to modulate cell proliferation, cell death, tumor invasion, and angiogenesis. As such, the inhibition and the targeting of COX-2 has received attention as a means of treating certain cancers and improving drug delivery overall [129]–[132].

Chapter Two: Thesis Work

Stimuli-Responsive Nanoscale Hydrogels for the Improved Delivery of Chemotherapeutic Agents

As indicated by the literature review, due to their versatility and multifunctionality, nanoparticles show great promise in improving the efficacy of current therapeutics by overcoming the plethora of biological barriers to drug delivery present in the body. In this particular thesis work, the nanoparticles of interest are specifically pH-sensitive nanoscale hydrogels. Hydrogels are polymeric materials that swell in the presence of water while maintaining a definitive three-dimensional structure, and they were the first biomaterial to be designed for medical applications [133]. As previously described, engineered nanomaterials hold promise for overcoming the various limitations to conventional drug delivery for a litany of reasons including but not limited to the following: by altering the drug clearance rate and biodistribution, by improving drug solubility, by protecting the therapeutics during circulation, and by possessing the ability to deliver multiple agents at once to a specific target – thereby providing a synergistic therapeutic effect. However, the polymeric nanoparticle subclass of materials is an especially attractive option for cancer

therapy for additional reasons. Polymers have a favorable size distribution and a high drug carrying capacity. The molecular architecture of the gels can be precise controlled, while possessing a variety of chemical structures. The material properties (mechanical, surface, etc.) are tunable, and the surface can be easily functionalized with desirable features or moieties. Additionally, intelligent polymers that respond to biological cues are of great interest because of their ability to provide controlled release at a specific site [6]–[9].

The nanoparticles studied in this thesis and their delivery capabilities were assessed in the context of ovarian cancer. According to current American Cancer Society estimates, over 22,000 women are diagnosed with ovarian cancer each year, and unfortunately over 14,000 women die each year from the disease, making ovarian cancer the 5th leading cause of cancer related deaths among women [134]. Because early stage ovarian cancer rarely causes any symptoms, it can often be misdiagnosed. Therefore, it is very difficult to detect, often only observed once it has metastasized and entered the late stages of development – making it a particularly dangerous and often fatal form of cancer [135]. In fact, while the 5-year survival rate for early stage ovarian cancer is about 92%, only about 15% of all cases of the cancer are discovered in this stage, and the overall 5-year survival rate for all types of ovarian cancer is only about 47% [134]. Because of the severity of the problems associated with ovarian cancer, there is a strong motivation to improve upon the current therapies used in its treatment. Additionally, because various ovarian cancer cell lines are well-studied and used ubiquitously in cancer studies, it is a good model in which to study these nanogel materials and their delivery capabilities.

Prior Work on Designing Stimuli-Responsive Nanogels

In Dr. Nicholas Peppas' biomaterials laboratory, extensive polymerization and materials research has already been conducted regarding pH-sensitive hydrogel nanoparticles as delivery

agents for the chemotherapeutic drug, carboplatin. Multiple components comprise the nanogels and confer different properties necessary for their delivery purposes. The components include: **(i)** a cationic monomer, 2-(diethylamino) ethyl methacrylate (DEAEMA), which provides the nanoparticles with their pH-sensitivity, as well as the characteristic swelling property that allows for the drug's diffusion out of the hydrogels once stimulated; **(ii)** a hydrophobic monomer, cyclohexyl methacrylate (CHMA), which enables tunability of the nanoparticle pKa and is critical for the intelligent design of the pH-stimulated swelling response; **(iii)** a crosslinking agent, tetraethylene glycol dimethacrylate (TEGDMA), which gives the nanoparticles their 3-dimensional properties; and finally, **(iv)** a surface graft, poly(ethylene glycol) methacrylate (PEGMA), which serves as a hydrophilic stealth agent to protect the nanoparticles from opsonization during intravenous injection. The tunable pH-sensitivity and swelling properties of these nanoparticles has already been assessed in previous studies, showing that the nanogels remain collapsed under physiological conditions (pH ~ 7.2-7.4) and swell once internalized by an acidifying endosome (pH ~ 6.0-6.5).

The Problem: Internalization and Target Specificity

It has yet to be discovered, though, how these nanoparticles are internalized by the ovarian cancer cells, and how their composition and surface properties – most notably, those conferred by the stealth agent – affect internalization, in terms of uptake mechanism and rate. The efficacy of chemotherapeutic drugs depends upon their uptake by the cancer cells. Barriers to the drugs' internalization will hinder the intended therapeutic effect and could potentially lead to adverse symptoms. Therefore, it is crucial to understand the mechanism by which the nanoscale drug delivery agents are internalized and trafficked by the cells in order to more intelligently design the composition of the delivery agents for optimized uptake.

Furthermore, it has been observed that the vesicles created for different internalization mechanisms possess different environmental conditions and trafficking routes. In the cases of both clathrin-dependent and clathrin-independent endocytosis, the endosomes possess an acidic pH which gradually becomes more acidic as it differentiates into a mature endosome and finally binds with a lysosome. In these cases, the pH-responsive nanogels would swell by the "proton-sponge effect" and rupture the endosomes, leading to the release of the nanogels' cargoes into the cytoplasm [136]. On the other hand, in the case of caveolin-dependent endocytosis, the endosomes created possess a neutral pH. Under these conditions, the nanogels, as they are currently designed, would remain collapsed and would fail to deliver the loaded cargoes [40]. Therefore, in order to prove that these pH-responsive nanogels possess the potential to serve as effective controlled-delivery agents, the mechanism by which they are uptaken by cells must be determined. Once the mechanism is discovered, the surface properties of the nanoparticles could be tuned through ligand functionalization or surface modification for improved target specificity and, thus, drug efficacy.

If the mechanism of uptake for these nanogels is, in fact, one that will lead to acidification within the endosome, the pH-responsive nature of these nanogels will render them suitable for a controlled release of the loaded cargo once internalized by a cell, rather than during circulation. However, this tunability alone will not necessarily improve the therapeutic effect conferred by these nanocarriers. In fact, something must be done in order to make these nanogels target-specific for cancer cells, with minimal uptake among healthy tissues.

As previously described, it is desirable to have nanoparticles that are coated with PEG during circulation because the inclusion of a stealth agent will prevent the removal of the nanoparticles and their cargoes from the body prematurely. However, it is expected that the inclusion of a hydrophilic stealth agent, like PEG, will impede uptake. Therefore, it would prove

beneficial to design a nanoparticle that will shed the stealth agent surface graft once in the presence of cancerous tissues only. This additional ability to intelligently respond to environmental cues will not only improve the target specificity of these nanocarriers for cancer cells but will also facilitate improved cellular uptake and thus drug efficacy.

In order to design such a stimuli-responsive coating, the unique qualities of the tumor extracellular environment must be exploited. One potential route for such an exploitation is to design an enzyme degradable cross-linker that functionalizes the PEG to the surface of the nanogels and that only degrades in the presence of a tumor-specific enzyme or an enzyme that is upregulated in cancerous tissues. Cathepsin B is a lysosomal cysteine protease primarily responsible for intracellular protein catabolism [137]. However, in a multitude of cancer types, including ovarian cancer, Cathepsin B has been shown to contribute to the growth and proliferation of tumor cells [138]. While it is confined to lysosomes in healthy tissues, Cathepsin B has been shown to be upregulated in cancer and often secreted from the tumor cells through membrane vesicle shedding. Upon secretion, the enzyme associates with the cell membrane surface and remains within the tumor microenvironment. Cathepsin B then degrades the surrounding extracellular matrix components – thereby facilitating tumor invasion and proliferation [139].

Cathepsin B is known to cleave numerous peptide sequences. Recently, there have been studies regarding Cathepsin B-cleavable doxorubicin prodrugs for improved targeted cancer therapy. One peptide sequence in particular – the tetrapeptide, Gly-Phe-Leu-Gly (GFLG) – has been found to be one of the most suitable cleavable spacers in the doxorubicin-based prodrugs for two main reasons: its plasma stability and its rapid hydrolysis in the presence of Cathepsin B. The enzyme degradable crosslinker, GLFG, has been used in the creation of numerous doxorubicin-based prodrugs – one of which, HPMA copolymer-Gly-Phe-Leu-Gly-doxorubicin, is already in

phase I/II clinical studies [140]. Therefore, GFLG will prove to be an ideal enzyme degradable crosslinker to use in the functionalization of PEG to the current nanogel design in order to assess the effect of PEG-shedding on cellular uptake.

Ultimately, there are three particular aims of this thesis work: (1) to elucidate the mechanism by which these nanogels are uptaken by cancer cells, (2) to evaluate how the surface properties of these nanogels – i.e. the inclusion of a stealth surface graft, PEG – affect uptake, and (3) to evaluate the effect of including a stimuli-responsive coating on cellular uptake. With this data, the nanogels can be further optimized in their design and composition for improved target specificity and thus drug efficacy.

Methodology

Materials

Poly(ethylene glycol) methyl ether methacrylate (Mn 2080) solution in 50 wt% in water (PEGMA), 2-(diethylamino)ethyl methacrylate (DEAEMA), tetraethylene glycol dimethacrylate (TEGDMA), cyclohexyl methacrylate (CHMA), myristyltrimethylammonium bromide (MyTAB), and the GGGG and GFLG tetrapeptides were purchased from Sigma-Aldrich (Sigma-Aldrich Corporation, St Louis, MO). Irgacure 2959 was obtained from Ciba (Ciba Inc., Basel, Switzerland). Brij 30 and deuterium oxide (99.8 % D) were purchased from Acros Organics. Acetone, 1X Dulbecco's phosphate buffered saline (DPBS), hydrochloric acid, and sodium hydroxide were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA). OVCAR-3 (ATCC® HTB-161™) and RPMI-1640 Medium (ATCC® 30-2001™) were obtained from ATCC (ATCC, Manassas, VA). Insulin from bovine pancreas solution (10 mg/mL insulin in 25 mM HEPES pH 8.2, Catalog I-0516) was obtained from Sigma-Aldrich, and fetal bovine serum (Corning, Catalog 35010CV) was obtained from Thermo Fisher Scientific. Water

used in all experiments was deionized (DI) with a Milli-Q Plus Ultrapure Water System (Millipore) equipped with a 0.22 μm in-line outlet filter. All chemicals were used as received.

Procedure

These studies determined how the copolymer composition and particle properties played a role in cellular internalization.

First, the internalization mechanism and intracellular distribution of the nanoparticles was assessed for previously synthesized nanoparticles of the standard design. Cells used in this study were human ovarian carcinoma cells (OVCAR-3 from ATCC) because they have a high genetic similarity to ovarian tumors [141]. After passaging the cell line at least three times, numerous sets of cells were plated and exposed to different combinations of pharmacological inhibitors. Then, after a sufficient incubation period, these cells were exposed to the various types of fluorescently labeled nanoparticles, and the internalization was monitored by flow cytometry.

Second, a series of nanogels with varied composition and properties were synthesized. All nanogels were fluorescently labeled for use in subsequent cell studies. The formulations were varied in the level of PEG coating to determine the influence of the stealth coating on uptake. After synthesis and purification, the samples were characterized through a number of analytical methods. Nuclear Magnetic Resonance Spectroscopy (NMR) and Fourier-Transformed Infrared Spectroscopy (FTIR) were used to confirm that the nanoparticles had been successfully synthesized and possessed the desired compositions. Dynamic light scattering (DLS) characterized the nanoparticle swelling properties and size, while zeta potential characterized their surface properties. Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) were used to assess the polymer properties of the nanoparticles. Then, the uptake of the series of fluorescently labeled nanogels of varied PEG content was monitored by flow cytometry.

Third, two series of nanogels had their surfaces functionalized – one series with the active, Cathepsin-cleavable crosslinker Gly-Phe-Leu-Gly (GFLG) and the other series with the non-active crosslinker Gly-Gly-Gly-Gly (GGGG). The crosslinkers were attached to PEG (MW:2000) and to the nanogel surfaces through an EDC/Sulfo NHS covalent coupling procedure. Samples for each nanogel series ranged from 0-20% molar PEG content to further assess the effect of PEG content on uptake, as well as the ability of the enzyme to degrade the crosslinker depending on steric hindrance. The samples with the inactive peptide crosslinker served as controls for comparison. These nanogels, once again, were fluorescently labeled, and uptake was monitored using flow cytometry.

Nanogel Synthesis. Nanogels with varying composition were synthesized using a well-known oil-in-water emulsion UV-initiated polymerization [142]. The amount of PEGMA was varied from 0 to 18 molar percent. The comonomer feed was comprised of DEAEMA, CHMA, and PEGMA. Irgacure 2959 was used as a photo-initiator, TEGDMA was used as a crosslinking agent, and Brij30 and MYTAB served as emulsifiers. After reacting for 2.5 hours under UV light, unreacted monomers and surfactants were removed by ionomer collapse. After purification, the nanogels were fluorescently labeled with Oregon Green 488 carboxylic acid succinimdyl ester (OG488).

Nuclear Magnetic Resonance Spectroscopy (NMR). Nanogel composition after synthesis was confirmed using 1H Proton NMR Spectroscopy. Nanogels were dissolved in deuterated-dimethyl sulfoxide at a concentration of 0.5 mg/mL for testing.

Fourier Transform Infrared Spectroscopy (FTIR). Nanogel composition after synthesis was further confirmed using FTIR. Dry powder samples were produced through lyophilization for 48 hours which were then used on the FTIR. Background spectra were

collected immediately before each sample and used for background subtraction. In all cases, spectra were averaged over 64 scans with 0.482 cm^{-1} data spacing.

Dynamic Light Scattering (DLS) and Zeta Potential. The nanogel particle size, swelling response, and surface charge were characterized by DLS and zeta potential. The particles were measured in phosphate buffered saline as a function of pH from 4.0 - 10.0 at a concentration of 0.5 mg/mL. To characterize the swelling response, the swelling onset and critical pHs were also calculated.

Differential Scanning Calorimetry (DSC). The nanogels were lyophilized for 48 hours and then characterized with differential scanning calorimetry (DSC) using a TA Instruments DSC-Q2000 instrument equipped. Samples were analyzed using Tzero aluminum pans and hermitic lids, with an empty pan and lid used as a reference. To determine thermal transitions, 5 mg of sample was transitioned from -70ºC to 350ºC in a heat/cool/heat cycle: (i) first ramping 10ºC per minute from 25ºC to 130ºC, (ii) followed by 5ºC per minutes to -70ºC, and (iii) followed by 10ºC per minute to 400ºC.

Thermogravimetric Analysis (TGA). The nanogels were lyophilized for 48 hours and then characterized with thermogravimetric analysis (TGA) using a TA Instruments TGA-Q500 instrument with platinum pans. To determine the decomposition profile, 5 mg of sample was first equilibrated to room temperature under nitrogen, subsequently heated 10ºC per minute to 550ºC under nitrogen, and followed by an equilibration at 550°C for 5 minutes.

Nanogel Fluorescent Labeling: Fluorescent labeling of nanoparticles using nanoparticles containing primary amines reacted with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride) in ethanol. NBD chloride reacts with primary amines to form fluorescent NBD-amines. The

primary amine, in these cases, was amino ethyl methacrylate (AEMA) which was covalently conjugated to the surface of the nanogels during UV polymerization. After reaction of the NBD chloride with the nanogels, the unreacted dye was removed from the nanogel samples through sequential rounds of dialysis, and then the samples were placed in the dark until use.

Ovarian Cancer Cell Culture. Cell studies used human ovarian carcinoma, CD44 overexpressing cells (OVCAR-3 from ATCC) because they have a high genetic similarity to ovarian tumors [141]. Cells were maintained using the ATCC protocol with RPMI-1640 medium (ATCC) supplemented with 0.01 mg/mL Bovine Insulin and 10% Fetal Bovine Serum. Media was changed every two days, and cells were passaged and sub-cultured when they reached 80-90% confluency.

Mechanism of Nanogel Uptake. Several chemicals were added to the cell culture wells in order to inhibit specific mechanisms of uptake. Filipin III was used to inhibit caveolar-dependent pathways [58]; Dynasore was used to inhibit clathrin-dependent endocytosis [143]; and Chlorpromazine was also used to inhibit clathrin-dependent endocytosis, while having no effect on clathrin-independent endocytosis, allowing the removal of the confounding factors associated with Dynasore, which, as a dynamin-inhibitor, prevents some clathrin-independent endocytosis, as well [58]. Amiloride was used to inhibit macropinocytosis; Wortmannin was used to inhibit phagocytosis, as well as macropinocytosis; and finally, one plate of cells was placed in a 4^{o}C environment in order to inhibit energy-independent pathways [144], [145].

EDC/Sulfo NHS Covalent Coupling. PEG(2000)-NHS (pre-activated) was reacted with the two tetrapeptides, GGGG and GFLG, at the N-terminals to create PEG-peptide conjugates. Each of the PEG-peptide conjugates was reacted at the C-terminal with N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide (i.e. EDC) to produce an Acylisourea intermediate,

which in the presence of N-Hydroxysuccinimide (NHS) created an amine-reactive Sulfo-NHS ester. Finally, these intermediates were reacted with primary amines on the surface of the nanogels to create a stable amide bond and thus PEG-peptide-nanogels. The quantity of PEG-peptide reacted varied for each sample in order to create a series of nanogels with varied surface conjugations. 0, 5, 10, 15, and 20 molar % PEG samples were created for both the GGGG and GFLG sample series [146].

Flow Cytometry. Flow cytometry was used to quantify nanoparticle internalization in the ovarian cancer cells (OVCAR-3). Measurements were collected using a BD Accuri Flow Cytometer and analyzed using both ForeCyt and FACSDiva software (IntelliCyt, BD Biosciences). The results are reported as the average percent of cells (taken over a large number of cells, typically 10,000) containing fluorescently-labeled nanoparticles, and all results will be reported as the average ± the standard deviation of six independent experiments.

Results and Discussion

Aim 1: In accordance with the first aim, the uptake of the standard nanogel design by ovarian cancer cells in the presence of various pathway inhibitors was evaluated in order to determine the mechanism by which the nanogels are internalized. The cellular uptake data can be found in **Figure 2**. In the presence of Wortmannin and Amiloride, the nanogel internalization was not inhibited, suggesting that the cells do not implore macropinocytosis or phagocytosis for the nanogel uptake (mechanisms more often reserved for particles in the micron size range). Additionally, Filipin III failed to inhibit uptake, and it can thus be concluded that the nanogels are not internalized by caveolar-dependent pathways. Nanogel uptake was significantly inhibited by incubation of the cells at 4^{o}C, suggesting that at least a portion of the nanogels are internalized by an energy-dependent pathway. Though, because uptake was not completely inhibited, the nanogels

must be primarily uptaken by an energy-independent pathway. Cellular internalization was much more severely inhibited by both Chlorpromazine and Dynasore. Therefore, the OVCAR cells must internalize the nanogels by a clathrin-dependent mechanism. As such, the nanogels will be enveloped by acidic vesicles that gradually differentiate into mature endosomes. This acidification will result in the swelling of gels by the "proton sponge" effect, the rupture of the endosomes, and the successful delivery of the nanogel cargo to the cell cytoplasm.

Aim 2: In the pursuit of the second aim of this thesis work, a series of nanogels were synthesized with varied PEG molar content ranging from about 0-18 mol%. Numerous characterization and analytical techniques were performed in order to confirm that the particles were synthesized as designed and to assess the effect of increasing PEG content on the polymer properties. The analytical data can be found in **Figure 3** - **Figure 7**.

^{1}H NMR and FTIR were used to confirm that the PEG was successfully incorporated into the nanoparticles. By comparing the ^{1}H NMR spectra for the linear polymer and the nanoparticle form in **Figure 3**, it was confirmed that PEG incorporated into the nanoparticles was primarily grafted to the surface of the nanoparticles, as intended. Furthermore, the ^{1}H NMR spectra in **Figure 4** exhibits an increasing PEG peak at 3.6 ppm, indicating the successful controlled incorporation of PEG. Additionally, the spectra also possess the characteristic nanoparticle peak at 1.3 ppm (illustrated in **Figure 3**, as well). The FTIR data presented in **Figure 5** illustrates the increase in PEG content for the nanoparticle series by the increasing intensity and definition of the ester C-O-C stretching peak at 1150 cm^{-1}, the C-H stretching peak at 1350 cm^{-1}, and the C-H bending peak at 2900 cm^{-1}.

TGA, DSC, and zeta potential were used to assess the effect of increasing PEG content on the polymer properties of the nanogels. The TGA data presented in **Figure 6** showed that the

fraction of mass lost between 180 and 285ºC decreased as the PEG content of the nanoparticles increased. This trend can be attributed with a decrease in the amount of water lost to anhydride formation due to the decrease in the quantity of esters present in the samples that contained higher PEG content. The DSC data presented in **Figure 6**, as well, showed a PEG melting endotherm that increased in intensity for samples that contained increasing PEG content. Additionally, as the PEG included in the monomer mix prior to polymerization increased, the melting point for the synthesized nanoparticles approached that of a PEG homopolymer, 54ºC, further substantiating controlled incorporation of PEG in the nanoparticles. The zeta potential data for the nanogel series can be seen in **Figure 7**. As the cationic core of the nanogels becomes more shielded with an increase in the neutral PEG on the surface of the particles, the surface charge decreases and approaches neutrality across the pH range of ~ 3.5-10.5. Additionally, the isoelectric point for all nanogels, regardless of ligand density, remained the same. This observation suggests that it is the nanogel core composition that determines the isoelectric point, and it further proves that the PEG content is segregated to the nanogel surface, as opposed to the internal core polymer network.

After synthesis and characterization, the nanogels of varied PEG content were fluorescently labeled and incubated with ovarian cancer cells (OVCAR-3) for different time periods (2, 24, 48, and 72 hours) in order to assess the effect of the nanogel surface properties, as well as time, on uptake. The cellular uptake data can be found in **Figure 8**. As expected, as PEG content increased, cellular internalization of the nanogels diminished sharply on all time scales, with nearly zero uptake for particles with 10 mol% PEG or greater. As PEG content increases, the cationic particle core becomes more shielded, and the surfaces of the nanogels become neutrally charged, as well as increasingly hydrophilic – thereby impeding the interaction of the nanogels with the cell membranes. As previously described, while the PEGylation of the nanoparticle

surface can improve its ability to act as a drug delivery agent by preventing protein adsorption and improving circulation as well as extravasation, a significant stealth coating ligand density on the nanoparticle surface can also interfere with target cell interactions and thus uptake.

Aim 3: After evaluating the effect of the nanogel surface properties on uptake, in accordance with aim 3, the nanogels with enzyme degradable crosslinkers functionalizing the PEG chains to the nanogel surfaces were synthesized and analyzed to ensure successful synthesis. The analytical data can be found in **Figure 9** - **Figure 16**.

The ^{1}H NMR data presented in **Figure 9** illustrates that the PEG-peptide conjugate samples contain both the PEG peaks and the respective characteristic peptide peaks, indicating successful synthesis of the PEG-peptide intermediates via the carbodiimide coupling reaction. The ^{1}H NMR data presented in **Figure 10** and **Figure 11** similarly contain the necessary PEG, peptide, and nanoparticle peaks, proving successful conjugation of the respective PEG-peptide intermediates to the nanoparticles. Additionally, the increasing intensity in the PEG peak in both sets of ^{1}H NMR data evidences successful controlled grafting of increasing amounts of PEG to the nanoparticle surfaces. The FTIR data in **Figure 12**, similar to that of the previous nanoparticle FTIR spectra, shows the increase in PEG content for both series of PEG-peptide-nanoparticle conjugates by the increasing intensity and definition of the ester C-O-C stretching peak at 1150 cm^{-1}, the C-H stretching peak at 1350 cm^{-1}, and the C-H bending peak at 2900 cm^{-1}.

TGA and DSC were used to assess the effect of increasing PEG content and the inclusion of the peptide linkers on the polymer properties of the nanogels. The TGA data presented in **Figure 13** and **Figure 14** showed that the fraction of mass lost between 180 and 285^{o}C decreased as the PEG content of the nanogels increased – similar to that of the previous TGA data and for the same reason. In comparing the TGA data of the nanogels with and without peptide linkers, it can be seen

that the peptide linkers have little to no effect on the polymer properties of the nanogels. The DSC data presented in **Figure 13** and **Figure 14**, as well, showed a PEG melting endotherm that increased in intensity for samples that contained increasing PEG content. Additionally, the melting point for the PEG-peptide-nanoparticle conjugates approached that of a PEG homopolymer, 54^{o}C, similar to that of the previous DSC data, further showing the successful incorporation of PEG and the absence of a significant effect of the inclusion of a peptide linker on the overall polymer properties.

DLS was also performed on the PEG-peptide-nanoparticle conjugates and the data comparing the size and PDI of the original nanoparticle (before PEG-peptide conjugation) and the PEG-peptide-nanoparticle conjugates can be found in **Figure 15**. There exists a large disparity in size between that of the starting nanoparticle and the conjugates, further proving successful functionalization of the surface, as the PEG layer thickness contributes to the measured size. Additionally, as the PEG content on the nanoparticle surface increases, the measured size of the nanoparticles increases. This trend is a result of the PEG chains changing from a "mushroom" conformation (coiled and condensed) to a more "dense brush" confirmation (elongated) as the ligand density and steric hindrance on the surface increases. This size data, in conjunction with the constant PDI observed, further suggests successful controlled PEG incorporation on monodisperse nanoparticles.

Zeta potential measurements were made for the original nanoparticle (before PEG-peptide conjugation) and the PEG-peptide-nanoparticle conjugates in acidic pH, with and without the presence of the enzyme, Cathepsin B, and the data can be found in **Figure 16**. For both sets of samples – those with the GGGG and with the GFLG peptide linkers – the PEG-peptide surface functionalization shielded the cationic surface of the particle, leading to a decreased effective

surface potential, relative to that of the starting nanoparticle. Furthermore, as the surface functionalization increased, the shielding increased, leading to the observable downwards trend in measured surface potential for both sets of surface modified nanoparticles. Based on the negligent change in surface charge for the GGGG peptide crosslinker while in the presence of Cathepsin B, it can be concluded that the peptide used is, in fact, non-responsive. On the other hand, for the samples with the GFLG peptide crosslinker, while in the presence of Cathepsin B, the surface potential returned to that of the original, unshielded nanoparticle – thereby proving that shedding occurred and that the GFLG peptide linker is, in fact, responsive and enzyme degradable for each ligand density used.

The surface modified nanoparticles were incubated with ovarian cancer (OVCAR-3) cells both with and without the presence of Cathepsin B in order to determine if the use of the enzyme degradable crosslinker would facilitate improved uptake. The cellular uptake data can be found in **Figure 17**. It was observed that the non-PEGylated nanoparticles (the starting, control nanoparticles) possessed a significant degree of internalization. The PEGylated particles with peptide linkers had nearly zero uptake without the presence of the enzyme. However, when the enzyme was present, the nanoparticles with the responsive linker had uptake that resembled that of the non-PEGylated nanoparticles, while the nanoparticles with the non-responsive linker maintained negligible levels of uptake. This data suggests that the cleaving of the responsive linker by the enzyme successfully facilitated shedding of the conjugated PEG and thus improved uptake. It also further substantiates the previously attained data that suggested that the presence of PEG could potentially inhibit uptake – proving the necessity of including a responsive linker in the design of this hydrogel-based drug delivery system.

Conclusion

All three aims of this thesis work were achieved. Using the pathway inhibition technique, it was determined that the standard P(DEAEMA-co-CHMA)-g-PEGMA based pH-sensitive nanoparticle design was internalized by a clathrin-dependent mechanism. As such, upon internalization, the nanoparticles will undergo acidification within the endosome as it matures. This acidification will cause the swelling of the nanoparticles, due to their pH sensitivity, and the endosomes will rupture, releasing the nanoparticles and their cargoes into the cytoplasm as intended. By elucidating the mechanism, it has been proven that these nanogels show promise for controlled intracellular delivery.

After determining the mechanism of uptake, the effect of grafting PEG – a known stealth coating agent – to the surface of the nanoparticles on cellular uptake was evaluated. It was determined that increasing amounts of PEG led to diminished uptake as a result of decreasing surface charge and increasing hydrophilicity, both of which impede nanoparticle-cell membrane interactions. This data served as the motivation to pursue the third and final aim of this thesis work, which was creating a responsive linker that functionalized PEG to the nanoparticle surfaces. The responsive linker chosen was the tetrapeptide, Gly-Phe-Leu-Gly, for three main reasons: (1) its stability in plasma, (3) its rapid cleavage in the presence of Cathepsin B – an enzyme known to be overproduced in cancers and present in the extracellular matrix of tumors, (3) and its previous successes in the use of certain prodrug strategies. After synthesizing nanoparticles with PEG functionalized to their surfaces by this responsive linker, it was shown that, in the presence of the enzyme, the PEG shed from the nanoparticle surfaces and cellular uptake drastically improved.

Ultimately, a stimuli-responsive nanogel-based drug delivery system was designed with multiple functionalities. It can avoid clearance by the MPS during circulation by means of a stealth

coating surface graft – improving the loaded drugs half-life; it can then shed that stealth coating from the surface when in the presence of cancer cells specifically – thereby facilitating uptake and making the drug carrier more target specific; and it can provide controlled intracellular delivery by means of its pH-sensitivity. While there is certainly more that can be done to further improve upon the current nanogel design, this drug delivery system shows great promise as a platform for improving cancer treatments.

Future Work

This current nanogel design could be further developed by increasing its target specificity for cancerous cells – in this case, ovarian cancer in particular. One way to pursue this increased target specificity is to functionalize a ligand to the surface of the nanogels that would preferentially bind to a cell membrane receptor that is overproduced in ovarian cancer cells specifically. CD44 and its variants are transmembrane glycoproteins that serve as cell-adhesion receptors involved in multiple cellular processes, such as growth, differentiation, and motility [147]. In normal ovarian epithelial cells, though, the expression of CD44 is low, if not all together absent. However, it is shown to be expressed in the majority of epithelial ovarian carcinomas [148], [149]. CD44 has been shown to bind the hyaluronic acid present in the extracellular matrix of mesothelial cells – thereby mediating the adhesion of the ovarian carcinoma cells to the peritoneum and facilitating cancer progression and metastasis [149]–[152]. As such, it is possible to functionalize hyaluronic acid to the surface of the nanoparticles. Hypothetically, this functionality would lead to the preferential binding of the nanoparticles with ovarian cancer cells specifically, thereby facilitating and improving their uptake – further enhancing their promise as potent drug delivery systems.

As seen in this work, significant strides are being made in the field of nanomedicines to improve drug efficacy. However, the prospects for their use is actually a rather controversial issue

in industry. Some believe that the use of current nanomedicine technologies will not result in an improved therapeutic effect due to their low tumor accumulation efficiencies – some being even less than 1% [153]. Rather, administered nanoparticles and their cargoes tend to accumulate in other sites in the body, like the liver, spleen, and lungs [154]. Furthermore, the sheer number of biological barriers to successful drug delivery – as described in this thesis – clearly show the difficulties in designing effective nanomedicines. Though, that is not to say that nanomedicines do not still possess the potential to serve as effective therapeutics. For instance, it is noteworthy that, while the accumulation rates for these nanomedicines at the targeted site are low, they are, nevertheless, around 10-100 times more efficient than that of the bare drugs – indicating a clear improvement in the potential therapeutic margin of the administered drugs [153]. Therefore, it is not necessarily that an alternative to nanomedicines needs to be discovered. Rather, the approach to their design needs to be revamped to make their transition to clinical use more rapid and efficient; it is crucial that the creators of nanomedicines begin to reassess the scope of the problem.

One of the potential ways of redefining nanomedicine design is transitioning from a formulation-driven approach to a disease-driven development process. This top-to-bottom approach is rooted in understanding the biology of the specific target and using that knowledge to design a nanomedicine that will exploit the tumor pathophysiology and ensure the right efficacy of the drug. Overall, in this approach, there is a more well-defined challenge to overcome which will better facilitate the rational design of the nanomedicine. This approach will prove to be more successful than attempting to develop a delivery system and then amending it for a specific clinical problem, as is often the case in the field currently [155]. Regardless of approach, the design of novel targeted delivery systems is an arduous pursuit. However, through a concerted effort by scientists across the field of nanomedicine to consider all of the obstacles present in drug delivery,

it is certainly possible to innovate new efficacious nanomedicines, as seen in this work – potentially improving the quality of life for millions of people.

Figures

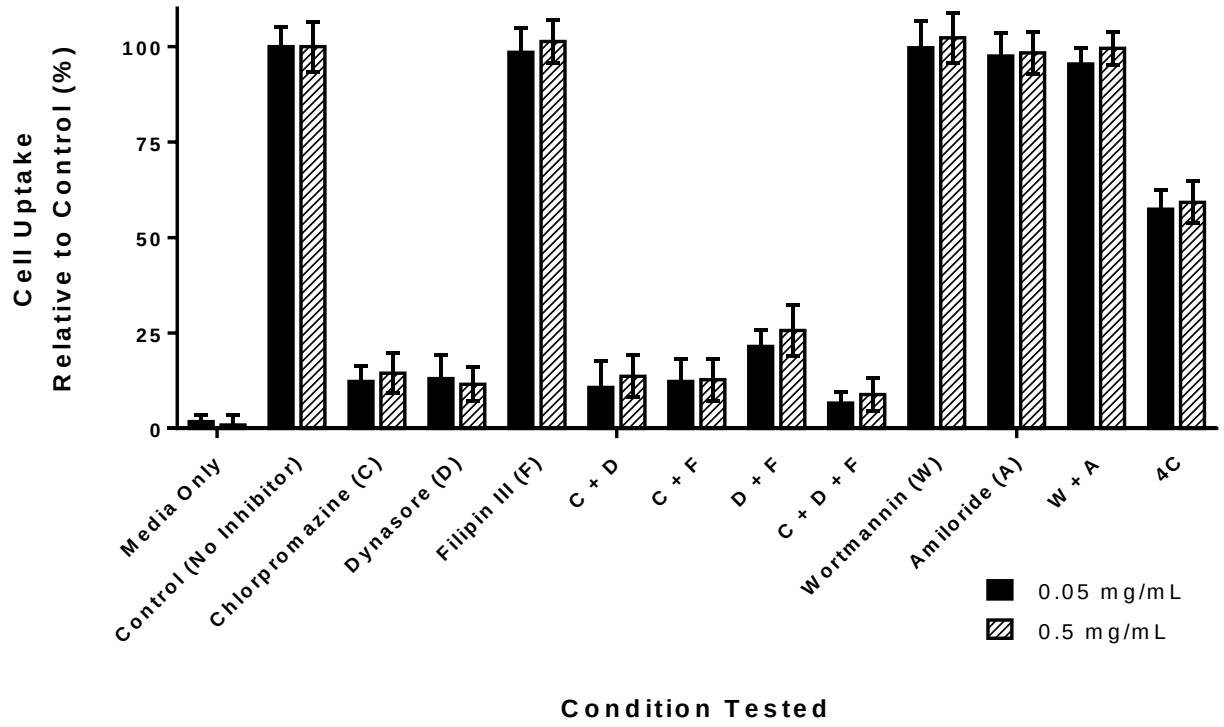

Figure 2. *Ovarian cancer (OVCAR-3) cellular uptake data for the current standard nanogel design in the presence of various pathway inhibitors. OVCAR-3 cells were incubated with the denoted permutation and concentration of pathway inhibitors for 1 hour prior to a 24-hour nanoparticle incubation period.*

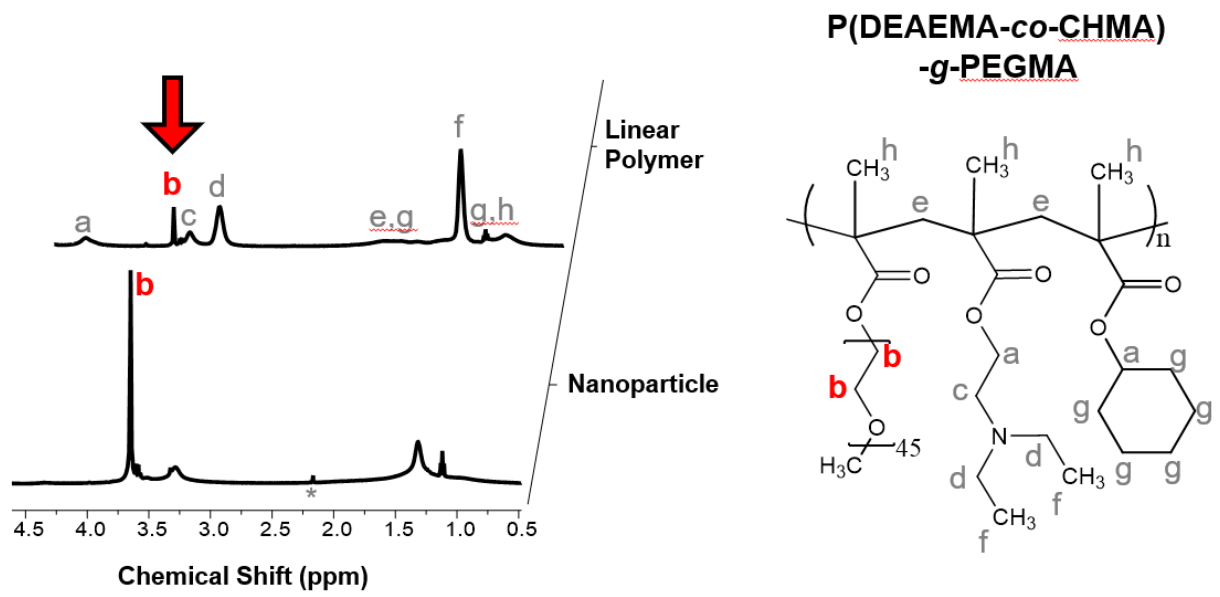

Figure 3. *A comparison of the ^{1}H NMR spectra for the linear polymer, P(DEAEMA-co-CHMA)-g-PEGMA, and the nanoparticle which includes the crosslinker, TEGDMA.*

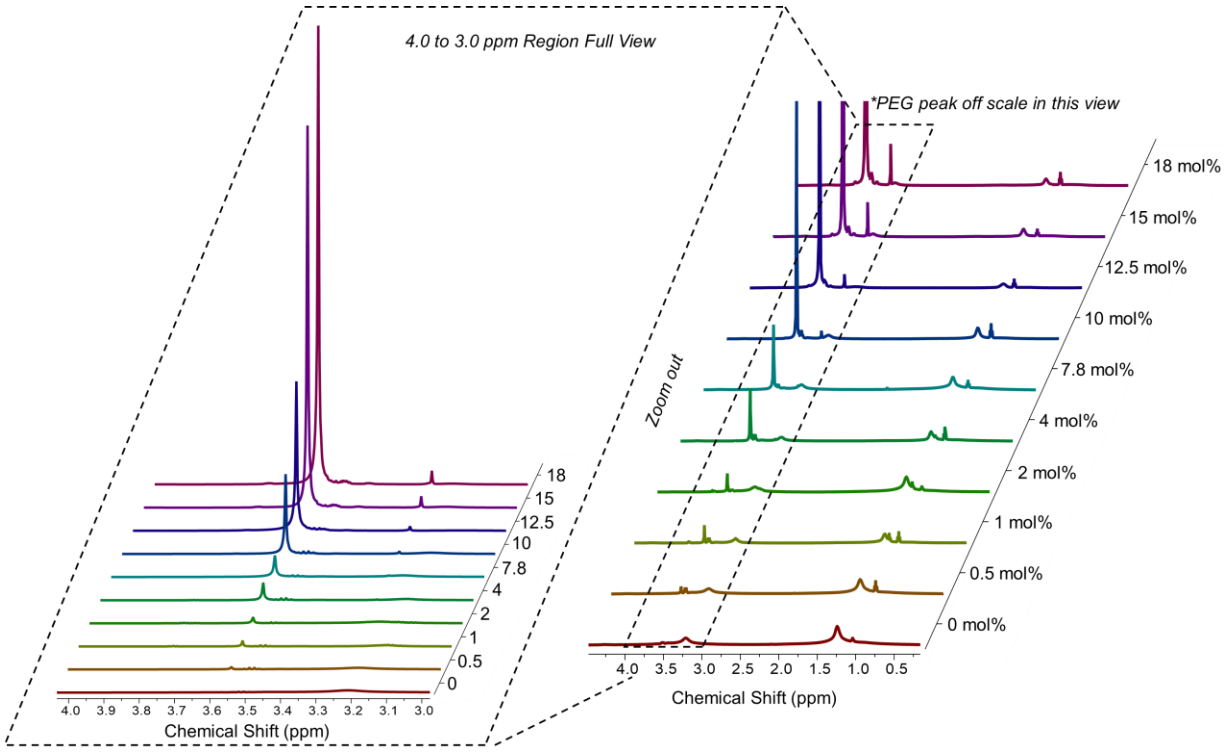

Figure 4. *^{1}H NMR of the nanogels synthesized with varied PEG content.*

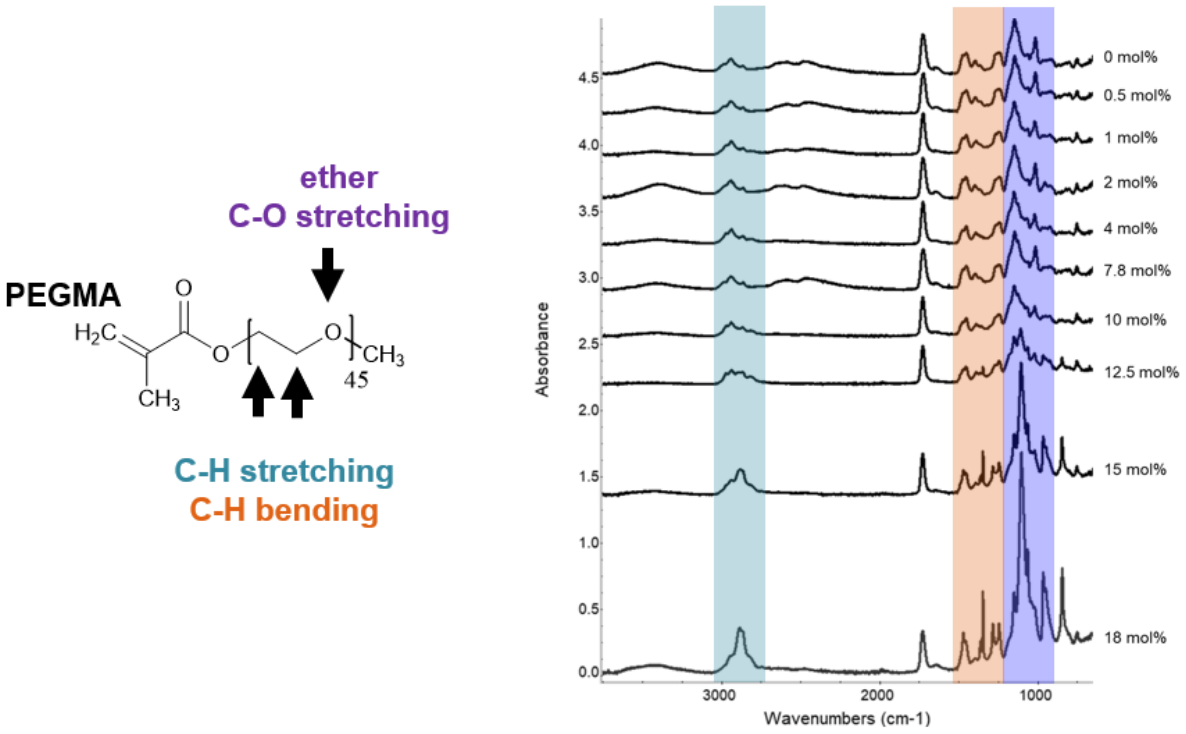

Figure 5. *FTIR of the nanogels synthesized with varied PEG content.*

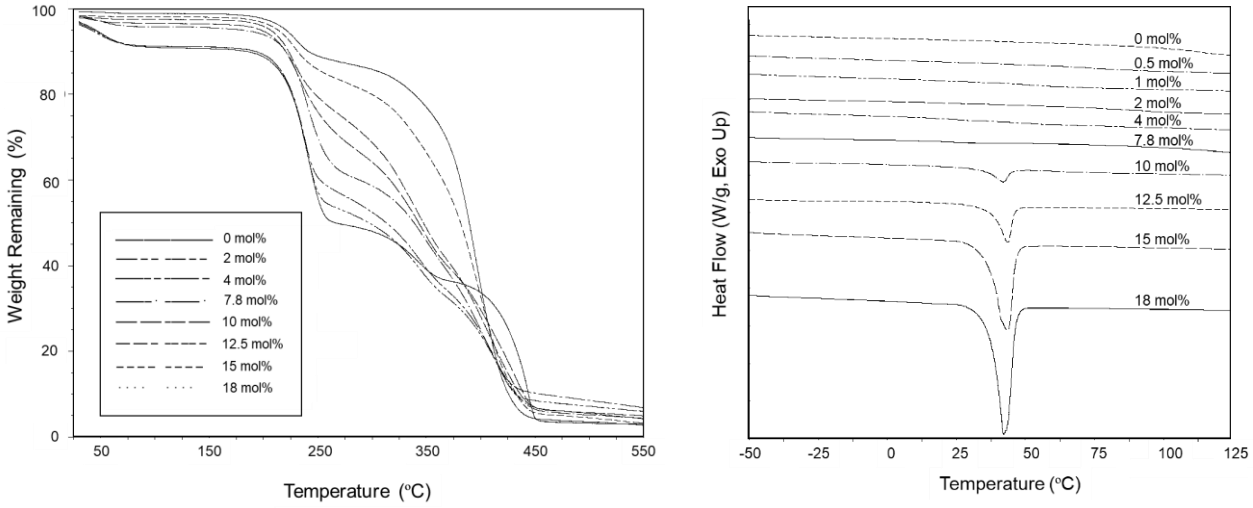

Figure 6. *Thermogravimetric Analysis (TGA-left) and Differential Scanning Calorimetry (DSC-right) of the nanogels synthesized with varied PEG content.*

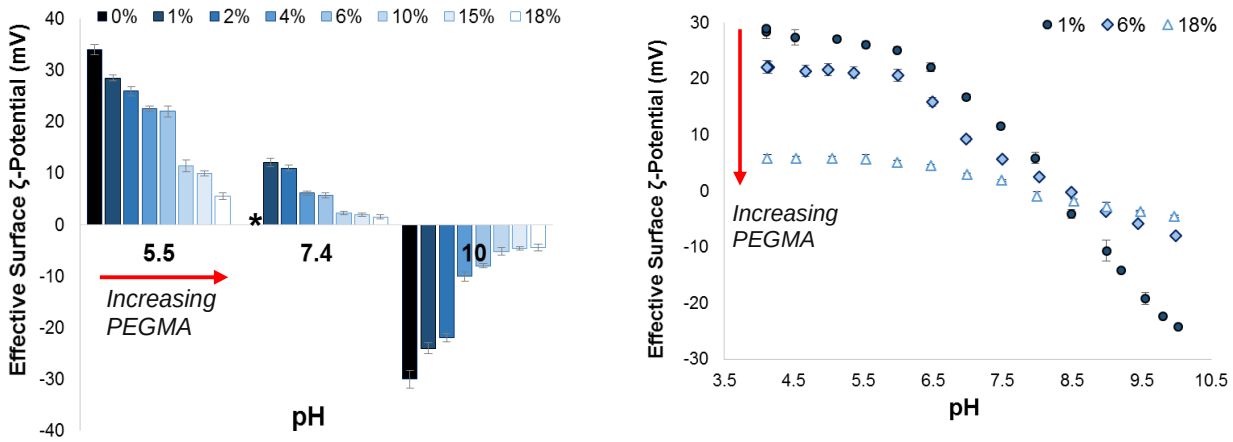

Figure 7. *Zeta potential measurement data for the nanogels synthesized with varied PEG content.*

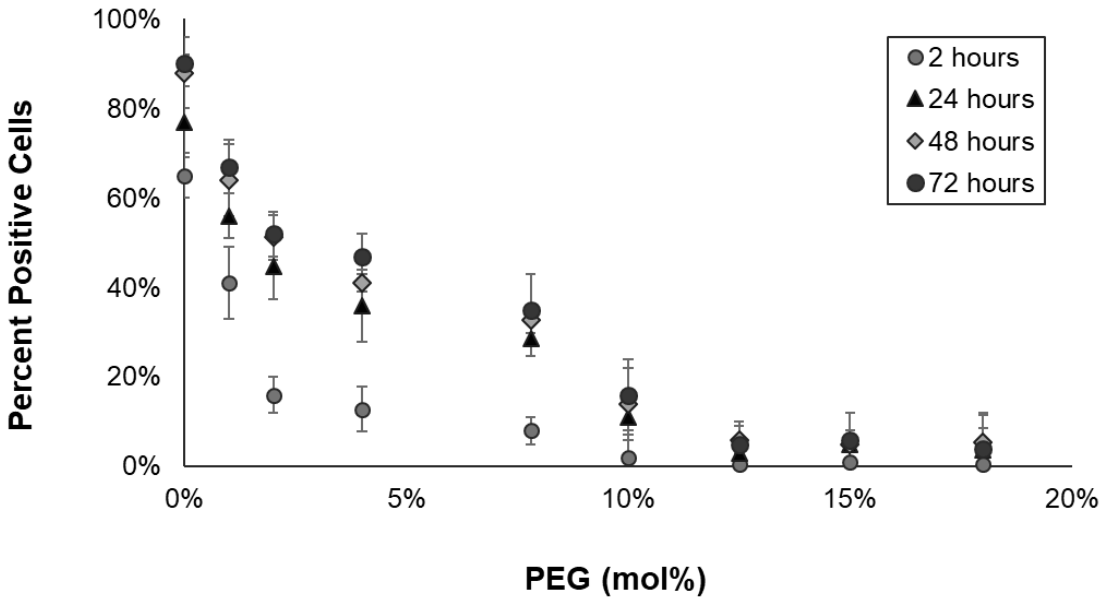

Figure 8. *Ovarian cancer (OVCAR-3) cellular uptake data for the series of nanogels synthesized with varied PEG molar content on the surface.*

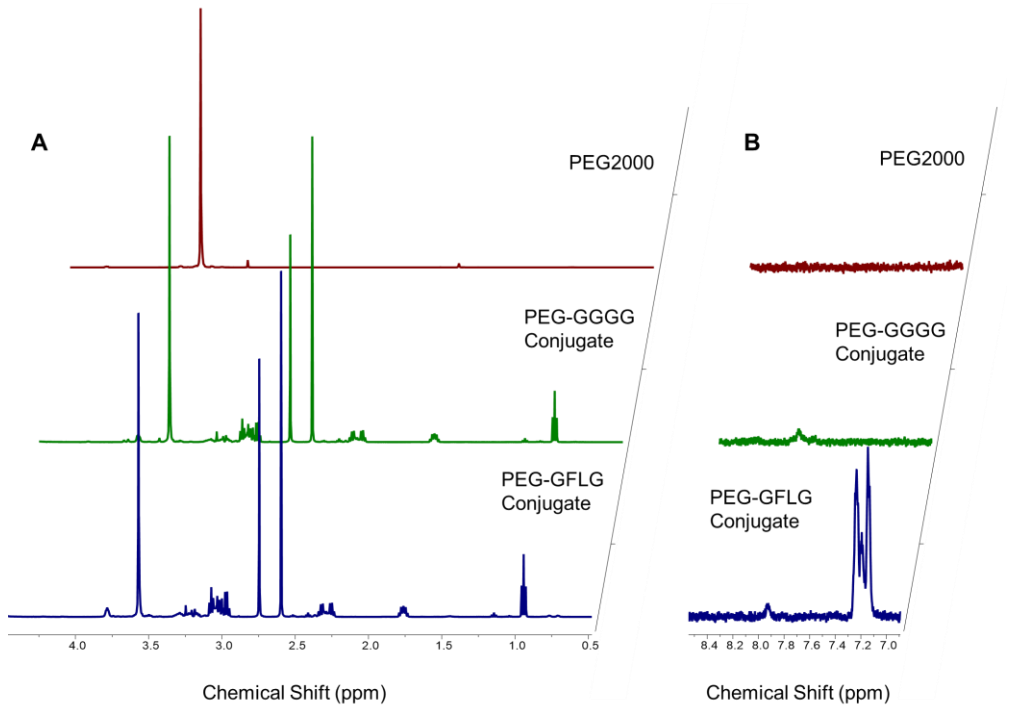

Figure 9. *^{1}H NMR for the PEG-GGGG and PEG-GFLG intermediates, as well as PEG2000 for comparison.*

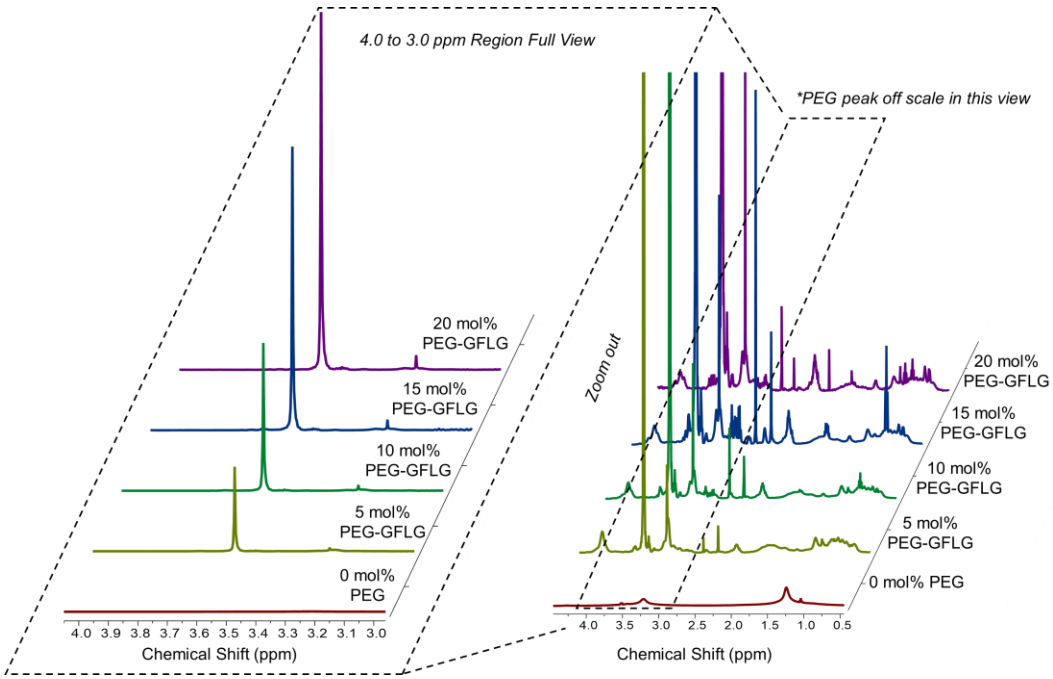

Figure 10. *^{1}H NMR for the PEG-GFLG-nanoparticle conjugates.*

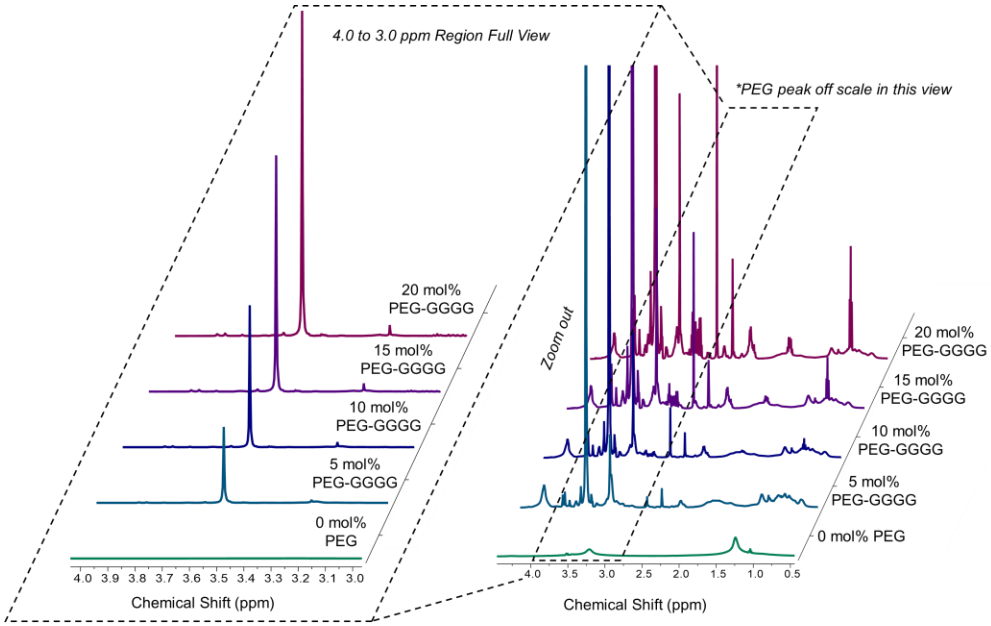

Figure 11. *^{1}H NMR for the PEG-GGGG-nanoparticle conjugates.*

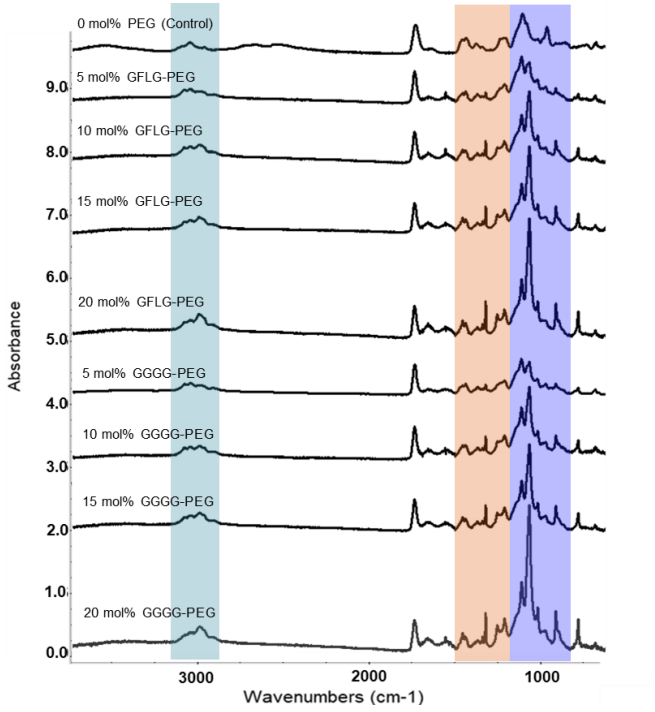

Figure 12. *FTIR data for the PEG-GFLG-nanoparticle (top) and PEG-GGGG-nanoparticle (bottom) conjugates with varied PEG content.*

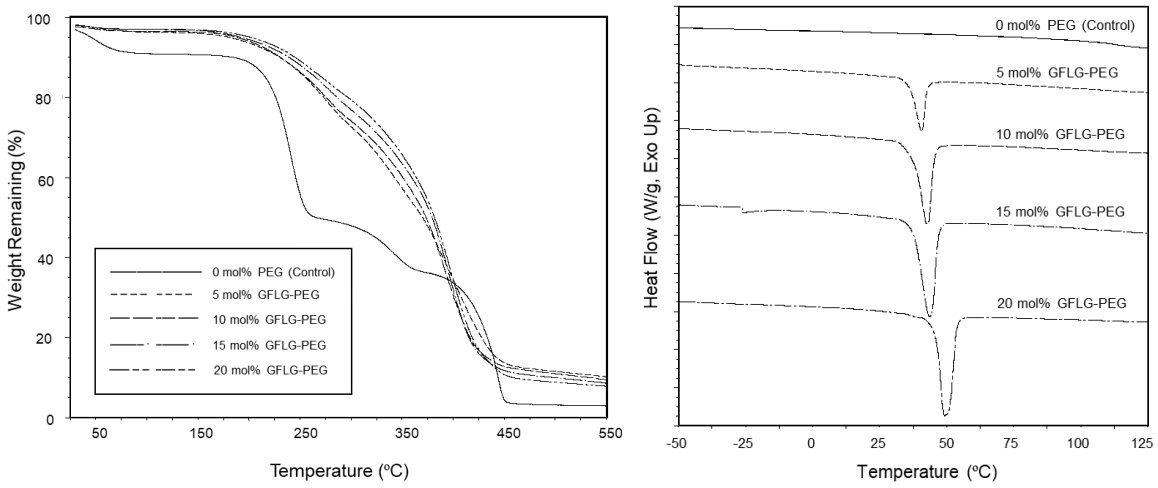

Figure 13. *Thermogravimetric Analysis (TGA-left) and Differential Scanning Calorimetry (DSC-right) data for the PEG-GFLG-nanoparticle conjugates with varied PEG content.*

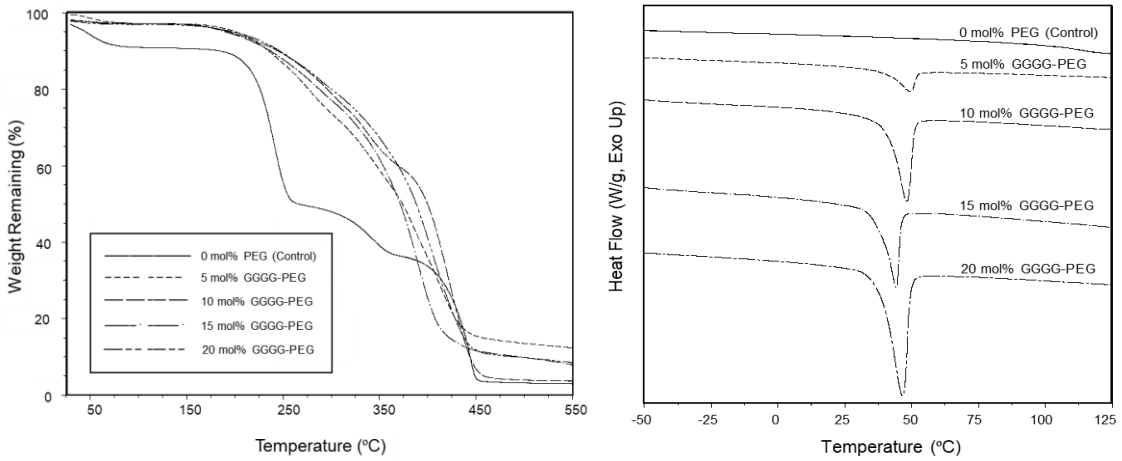

Figure 14. *Thermogravimetric Analysis (TGA-left) and Differential Scanning Calorimetry (DSC-right) data for the PEG-GGGG-nanoparticle conjugates with varied PEG content.*

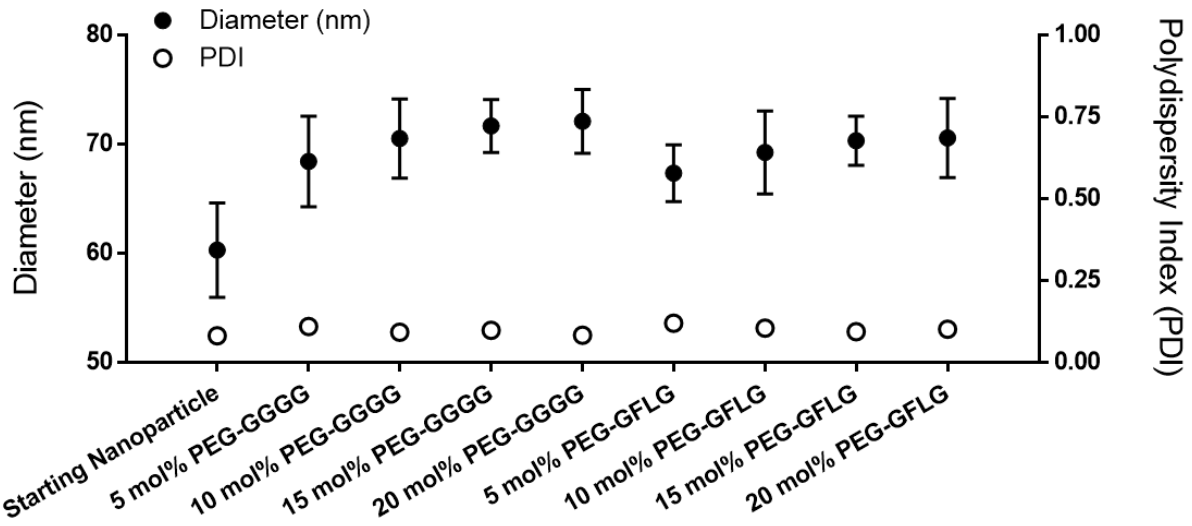

Figure 15. *DLS data comparing the size and PDI of the original nanoparticle (before PEG-peptide conjugation) and the PEG-peptide-nanoparticle conjugates.*

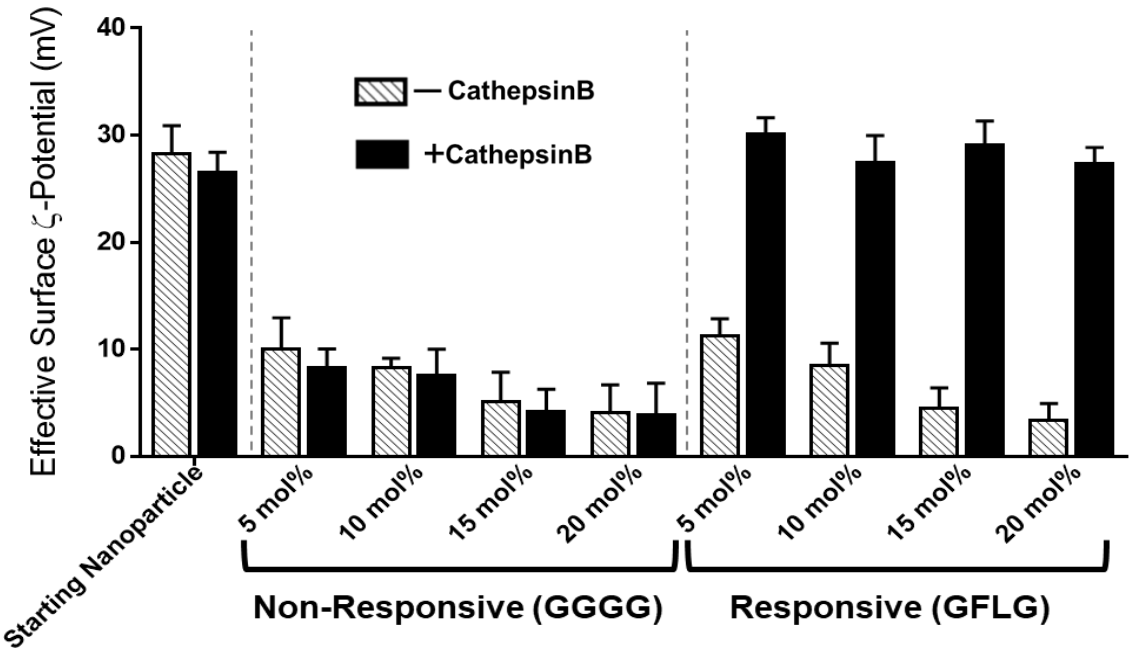

Figure 16. *Zeta potential data for the original nanoparticle (before PEG-peptide conjugation) and the PEG-peptide-nanoparticle conjugates in acidic pH, with and without the presence of the enzyme, Cathepsin B.*

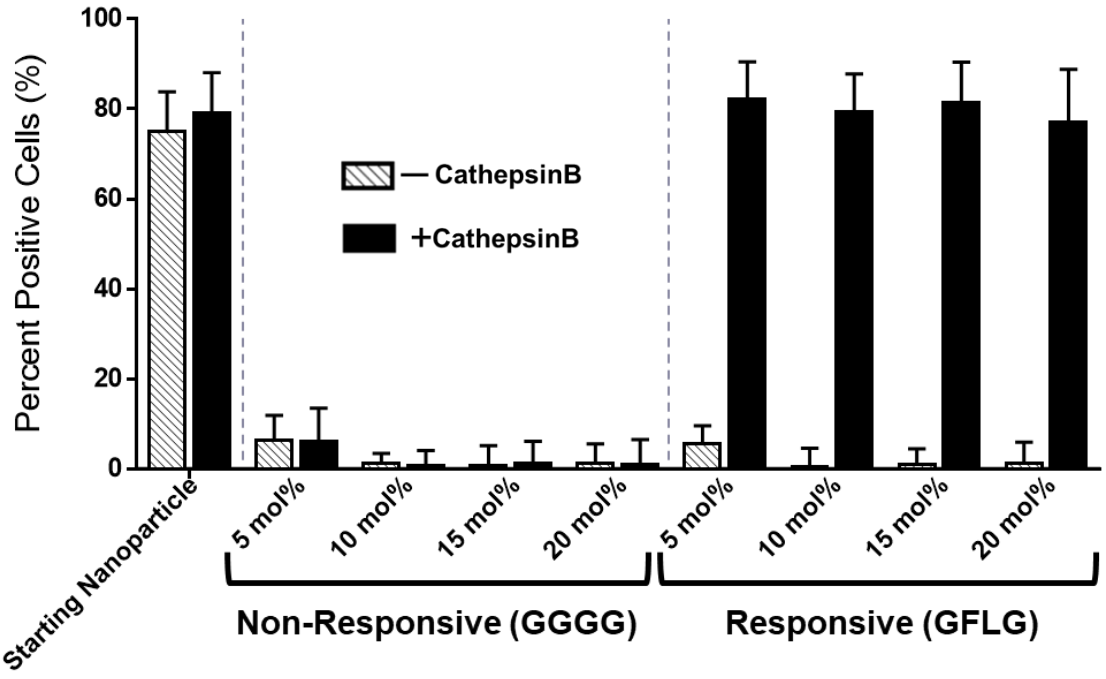

Figure 17. *Ovarian cancer (OVCAR-3) cellular uptake data for the original nanoparticle (before PEG-peptide conjugation) and the PEG-peptide-nanoparticle conjugates, with and without the presence of the enzyme, Cathepsin B.*

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